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(57) Abstract

The present invention provides gene therapy using recombinant vector delivery systems and particularly adenovirus vectors. The invention specifically provides replication-deficient vectors which are able to replicate specifically in certain abnormal tissues to provide a therapeutic benefit from the vector *per se* or from heterologous gene products encoded by the vector and distributed throughout the abnormal tissue. Preferably, the tissue is tumor tissue. The invention also provides cell lines for producing recombinant replication-deficient vectors useful for genetic therapy. The invention also provides methods for screening abnormal tissues, and especially tumors, for functions or functi nal deficiencies that permit vector replication.

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Tissue-Specific Treatment, Diagnostic Methods, and Compositions Using Replication-Deficient Vectors

Background of the Invention

Field of the Invention

The present invention relates to gene therapy using recombinant vector delivery systems and particularly adenovirus vectors. The invention specifically relates to replication-deficient vectors which are able to replicate specifically in certain tissues to provide a therapeutic benefit from the vector *per se* or from heterologous gene products encoded by the vector and distributed throughout the abnormal tissue. Preferably, the tissue is tumor tissue. The invention also relates to cells for producing recombinant replication-deficient vectors useful for gene therapy. The invention also relates to methods for screening abnormal tissues, and especially tumors, for functions or functional deficiencies that permit vector replication.

Background Art

Targeting Vectors

The introduction of exogenous genes into cells *in vitro* or *in vivo*, systemically or *in situ*, has been of limited use for mixtures in which it would be disadvantageous for non-target cells to take up the exogenous gene. One strategy to overcome this problem is to develop administration procedures or vectors that target a specific cell-type. Using systemic administration, attempts have been made to direct exogenous genes to myocytes and muscle cells by direct injection of DNA, to direct the exogenous DNA to hepatocytes using DNA-protein complexes, and to endothelial cells using liposomes.

Using *in situ* administration, retroviral replication functions have been utilized to target cells that are actively replicating. Targeting to replicatively

active cells in this manner has been augmented by incorporating "suicide" genes in the vector, which kill actively replicating untargeted, as well as targeted cells that take up the vector.

Thus far, the ability to target cells has been limited, however, by the lack of cell-type specificity and low gene transfer efficiencies. The limited ability to target an exogenous gene to diseased cells in an organism, while avoiding (eliminating) uptake of the gene by normal, untargeted cells, particularly has been an obstacle to developing effective gene-transfer-based therapies for diseases in animals and humans.

One especially difficult challenge is targeting tumor cells, because they have many of the functions of normal cells. Many seemingly promising strategies for these cells, moreover, are limited to one or a few cell-types. Thus, a way to limit vector uptake or viability to targeted cells remains to be developed.

The present invention, in one aspect, provides a way to deliver an exogenous gene in a tumor.

Adenoviruses Generally

Adenoviruses are nonenveloped, regular icosohedrons. The protein coat (capsid) is composed of 252 capsomeres of which 240 are hexons and 12 are pentons. Most of the detailed structural studies of the adenovirus polypeptides have been done for adenovirus types 2 and 5. The viral DNA is 23.85 x 10^6 daltons for adenovirus 2 and varies slightly in size depending on serotype. The DNA has inverted terminal repeats and the length of these varies with the serotype.

The replicative cycle is divided into early (E) and late (L) phases. The late phase defines the nset of viral DNA replication. Adenovirus structural proteins are generally synthesized during the late phase. Following adenovirus infection, host DNA and protein synthesis is inhibited in cells infected with most serotypes. The adenovirus lytic cycle with adenovirus 2 and adenovirus 5 is very

efficient and results in approximately 10,000 virions per infected cell along with the synthesis of excess viral protein and DNA that is not incorporated into the virion. Early adenovirus transcription is a complicated sequence of interrelated biochemical events, but it entails essentially the synthesis of viral RNAs prior to the onset of viral DNA replication.

The organization of the adenovirus genome is similar in all of the adenovirus groups and specific functions are generally positioned at identical locations for each serotype studied. Early cytoplasmic messenger RNAs are complementary to four defined, noncontiguous regions on the viral DNA. These regions are designated (E1-E4). The early transcripts have been classified into an array of immediate early (E1a), delayed early (E1b, E2a, E2b, E3 and E4), and intermediate (IVa2.IX) regions.

The E1a region is involved in transcriptional transactivation of viral and cellular genes as well as transcriptional repression of other sequences. The E1a gene exerts an important control function on all of the other early adenovirus messenger RNAs. In normal tissues, in order to transcribe regions E1b, E2a, E2b, E3, or E4 efficiently, active E1a product is required. However, as discussed below, the E1a function may be bypassed. Cells may be manipulated to provide E1a-like functions or may naturally contain such functions. The virus may also be manipulated to bypass the functions as described below.

The E1b region is required for the normal progression of viral events late in infection. The E1b product acts in the host nucleus. Mutants generated within the E1b sequences exhibit diminished late viral mRNA accumulation as well as impairment in the inhibition of host cellular transport normally observed late in adenovirus infection (Berkner, K.L., *Biotechniques* 6:616-629 (1988)). E1b is required for altering functions of the host cell such that processing and transport are shifted in favor of viral late gene products. These products then result in viral packaging and release of virions. E1b produces a 19 kD protein that prevents apoptosis. E1b also produces a 55 kD protein that binds to p53.

For a complete review on adenoviruses and their replication, see Horwitz, M.S., *Virology 2d ed.*, Fields, B.N., eds., Raven Press Limited, New York (1990), Chapter 60, pp. 1679-1721.

Adenovirus as Recombinant Delivery Vehicle

Adenovirus provides advantages as a vector for adequate gene delivery for the following reasons. It is a double stranded DNA nonenveloped virus with tropism for the human respiratory system and gastrointestinal tract. It causes a mild flu-like disease. Adenoviral vectors enter cells by receptor mediated endocytosis. The large (36 kilobase) genome allows for the removal of genes essential for replication and nonessential regions so that foreign DNA may be inserted and expressed from the viral genome. Adenoviruses infect a wide variety of cell types *in vivo* and *in vitro*. Adenoviruses have been used as vectors for gene therapy and for expression of heterologous genes. The expression of viral or foreign genes from the adenovirus genome does not require a replicating cell. Adenovirus vectors rarely integrate into the host chromosome; the adenovirus genome remains as an extrachromosomal element in the cellular nucleus. There is no association of human malignancy with adenovirus infection; attenuated strains have been developed and have been used in humans for live vaccines.

For a more detailed discussion of the use of adenovirus vectors for gene therapy, see Berkner, K.L., *Biotechniques 6*:616-629 (1988); Trapnell, B.C., *Advanced Drug Delivery Reviews 12*:185-199 (1993).

Adenovirus vectors are generally deleted in the E1 region of the virus. The E1 region may then be substituted with the DNA sequences of interest. It was pointed out in a recent article on human gene therapy, however, that "the main disadvantage in the use of adenovirus as a gene transfer vector is that the viral vector generally remains episomal and *does not replicate*, thus, cell division leads to the eventual loss of the vector from the daughter cells" (Morgan, R.A., *et al.*, *Annual Review of Biochemistry 62*:191-217 (1993)) (emphasis added).

Non-replication of the vector leads not only to eventual loss of the vector without expression in most or all of the target cells but also leads to insufficient expression in the cells that do take up the vector, because copies of the gene whose expression is desired are insufficient for maximum effect. The insufficiency of gene expression is a general limitation of all non-replicating delivery vectors. Because of this limitation, for example, the effective amount of ganciclovir used in conjunction with current thymidine kinase expression vectors results in unwanted side effects. However, if gene dosage is increased such that greater amounts of thymidine kinase are produced, less ganciclovir is required to produce the desired treatment and side effects are minimized. Thus, it is desirable to introduce a vector that can provide multiple copies of a gene and hence greater amounts of the product of that gene.

Published PCT Patent Application No. WO 94/18992 describes methods and compositions for treating neoplastic conditions based on the use of replication-deficient viruses in p53-minus or Rb-minus tumor cells. The publication asserts a virus able to produce a replication phenotype in neoplastic cells, but unable to produce a replication phenotype in non-replicating non-neoplastic cells having normal p53 and/or pRb function. The experimental data show cytopathic effects of the replication-deficient viruses but not the production of infectious virions. The reference does not disclose producer cell lines for vector production, and especially for virion production of recombinant viruses free of wild-type viruses. The reference does not disclose a combination of diagnosing a tissue type *ex vivo* for the ability to replicate a replication-defective virus and subsequent use of such virus to treat the tissue *in vivo*. The reference does not disclose methods and compositions wherein cellular functions other than p53 and/or Rb are deficient. The reference does not disclose replication of a replication-defective virus by means of complementing gene functions. To make a producer cell line or effective therapy involving the spread of the virus throughout the tissue, requires the production of infectious virus.

The present invention overcomes all of the disadvantages discussed above by providing a replicating vector, multiple DNA copies, and increased amounts of gene product, especially infectious virus.

Production of Adenoviral Vectors

Adenoviral vectors for recombinant gene expression have been produced in the human embryonic kidney cell line 293 (Graham, F.L. *et al.*, *J. Gen. Virol.* *36*:59-72 (1977)). This cell line, initially transformed with human adenovirus 5, now contains the left end of the adenovirus 5 genome and expresses E1. Therefore, these cells are permissive for growth of adenovirus 2 and adenovirus 5 mutants defective in E1 functions. They have been extensively used for the isolation and propagation of E1 mutants. Therefore, 293 cells have been used for helper-independent cloning and expression of adenovirus vectors in mammalian cells. E1 genes integrated in cellular DNA of 293 cells are expressed at levels which permit deletion of these genes from the viral vector genome. The deletion provides a nonessential region into which DNA may be inserted. For a review, see, Young, C.S.H., *et al.* in *The Adenoviruses*, Ginsberg, H.S., ed., Plenum Press, New York and London (1984), pp. 125-172.

However, 293 cells are subject to severe limitations as producer cells for adenovirus vectors. Growth rates are low. Titres are limited, especially when the vector produces a heterologous gene product that proves toxic for the cells, such as thymidine kinase. Recombination with the viral E1 sequence in the genome can lead to the contamination of the recombinant defective virus with unsafe wild-type virus. The quality of certain viral preparations is poor with regard to the ratio of virus particle to plaque forming unit. Further, the cell line does not support growth of more highly deleted mutants because the expression of E1 in combination with other viral genes in the cellular genome (required to complement the further deletion), such as E4, is toxic to the cells. Therefore, the amount of heterologous DNA that can be inserted into the viral genome is limited

in these cells. It is desirable, therefore, to produce adenovirus vectors for gene therapy in a cell that cannot produce wild-type recombinants and can produce high titres of high-quality virus. The present invention overcomes these limitations.

Tumor Suppressor Genes

Experiments in somatic cell genetics show that one or more genetic elements can inhibit tumor cell proliferation. These experiments suggested that genes contained in the normal cell suppress the ability of a normal cell to become tumorigenic.

Since the original experiments were done, two genes in particular have been characterized as tumor-suppressor genes, that is, genes that are able to suppress the tumorigenic phenotype in tumor cells. The retinoblastoma susceptibility gene (Rb) and p53 are two of the best studied of the tumor-suppressor genes. The inheritance of a defective allele of Rb predisposes to retinoblastoma, a childhood tumor of the eye. However, the Rb gene is also subject to somatic mutation in the development of breast, lung and bladder carcinomas, osteosarcomas, and soft-tissue sarcomas (Weinberg, R.A., *Cancer Surveys 12*:43-57 (1992)). p53 is mutated during the development of various sporadic human cancers, and germ-line mutations in p53 are associated with Li-Fraumeni syndrome (Harris, C.C. *et al.*, *New Engl. J. Med. 329*:1318-1327 (1993); Malkin, D., *Cancer Genet. Cytogenet. 66*:83-92 (1993); Malkin, D. *et al.*, *Science 250*:1233-1238 (1990)). Many human tumors show a high frequency of mutation of both p53 and Rb. Research on these genes has suggested that there may be cooperative tumorigenic effects of p53 and Rb in transformation. For a detailed review of these genes and their functions, see Levine, A.J., *Ann. Rev. Biochem. 62*:623-651 (1993).

Programmed cell death plays an important part in the regulation of development of multicellular organisms (see Ellis, R.E. *et al.*, *Ann. Rev. Cell*

Biol. 7:663-693 (1991) and Cohen, J.J. *et al.*, *Ann. Rev. Immunol. 10*:267-293 (1992)). Programmed cell death characteristically occurs by apoptosis, a process of nuclear condensation and cleavage of chromosomes into small DNA fragments. p53 appears to be an important component in the pathway towards apoptosis (Yonish-Rouach, C.D. *et al.*, *Nature 352*:345-347 (1991)). p53 can complex with viral and mammalian proteins. p53 can heterodimerize and form heterodimers with SV40 T-antigen, human papilloma virus E6 protein and adenovirus E1b. The binding of viral proteins to wild-type p53 either results in rapid degradation of p53 or in sequestration in an inactive form. The ability of viral proteins to inactivate p53 may be a mechanism by which these viruses transform cells (see Lee, J.M. *et al.*, *Mutation Res. 307*:573-581 (1994)). Accordingly, a block to programmed cell death may occur through the binding of a viral protein to p53 or by functional inactivation of p53 in a cell.

Cells that are resting in G_0 or are in G_1 have Rb protein which is found in a complex with a cellular transcription factor called E2F. E2F mediates the transcription of several viral genes as well as some cellular genes that contribute enzymatic activities involved in DNA replication. When the Rb/E2F complex is exposed to adenovirus E1a protein, the E2F protein is released from this complex, and there is an increased ability of E2F to promote the transcription of an E2F-responsive promotor element. Similarly, the HPV16 E7 protein interacts with Rb and releases E2F, providing a new activity. In this way, Rb acts during G_0 or G_1 in the cell cycle to sequester transcription factor E2F so that it will not transcribe a set of genes presumably required for entry into the S phase of the cell cycle. Rb may regulate a number of cellular proteins in this fashion (Levine, A.J., *Ann. Rev. Biochem. 62*:623-652 (1993)).

Accordingly, it would be desirable to have a method for ascertaining whether or not a given cell, and particularly whether a given tumor cell, is functionally inactivated in p53 or Rb.

Summary of the Invention

In view of the limitations discussed above, a general object of the invention is to provide a method for selectively distributing a vector *in vivo* in a tissue mass, and preferably in a tumor mass, such that a greater number of cells are treated than would be treated with a non-replicating vector, and treatment is avoided or significantly reduced in normal or non-tumor tissue.

Another object of the invention is to provide a method for identifying tumor cells that contain a factor(s) that allows replication of a replication-deficient vector or is deficient for a factor(s) that prevents replication of a replication-deficient vector. A preferable object of the invention is to identify tumor cells that do not contain functionally active p53 or p53 and Rb.

A third object of the invention is to provide producer cell lines for vector production, and preferably for adenovirus vector production. Preferably, the cell lines have one or more of the following characteristics: high titer virus production, resistance to toxic effects due to heterologous gene products expressed in the vector, lack of production of wild-type virus contaminating the virus preparation and resulting from recombination between cellular viral sequences and vector sequences, growth to high density and in suspension, unlimited passage potential, high growth rate, and by permitting the growth of highly deleted viruses that are impaired for viral functions and able to accommodate large pieces of heterologous DNA.

Accordingly, the invention is generally directed to tissue-specific gene therapy using a vector that selectively replicates only in a target tissue. The invention is specifically directed to a method for distributing a polynucleotide in a tissue *in vivo*, comprising introducing a replication deficient vector containing the polynucleotide into the tissue, allowing the vector to replicate in cells of the tissue, and in which cells the replication deficiency is complimented by one or more endogenous, naturally-occurring factors that allow the replication of the replication deficient vector in this cell or in which an endogenous, naturally-

occurring inhibitor of vector replication in functionally inactive in the cells, thereby allowing vector replication to occur. Thus, the invention is also directed to a method for distributing a vector in a target tissue and a method for distributing a gene product produced from a gene on the vector in a target tissue.

In a preferred embodiment, the tissue is abnormally proliferating, and especially is tumor tissue. However, the invention is also directed to other abnormal tissue as described herein, as well as normal tissue.

In a preferred embodiment, the replication-deficient vector is a DNA tumor viral vector. In preferred embodiments, the DNA tumor viral vector is a vector selected from the group consisting of herpesvirus, papovavirus, papillomavirus, and hepatitus virus vectors.

In a most preferred embodiment, the vector is an adenovirus vector.

In a further preferred embodiment, the E1 region, and preferably the E1a region, is deleted from the adenovirus.

In a further preferred embodiment of the invention, the cells are functionally inactive for tumor suppression gene products, and preferably, for at least p53, and more preferably, also for Rb.

In a further preferred embodiment, the cells contain functionally active p53 and Rb, but also contain an endogenous cellular factor(s) that inactivate at least p53 and preferably, also Rb. Such a function can arise from an endogenous cellular element(s) resulting from natural virus infection, such as an integrated provirus(es) that expresses proviral functions from the cellular genome that inactivate the functions that interfere with or prevent viral replication.

In a further embodiment of the invention, the vector encodes a heterologous gene product and expresses this heterologous gene product in the cells of the target tissue.

In a further embodiment of the invention, the heterologous gene product is toxic for cells in the target tissue.

In a further embodiment of the invention, the heterologous gene product acts on a non-toxic prodrug, converting the non-toxic prodrug into a form that is toxic for cells in the target tissue. Preferably, the toxin has anti-tumor activity.

In a further embodiment of the invention, the vector is introduced into the tissue by virus infection. In a preferred embodiment, the virus is an adenovirus.

Replication can be vector replication alone or can also include virus replication (i.e., virion production). Thus, either DNA or virions or both may be distributed in the target tissue.

In another embodiment of the invention, a method is provided for assaying vector utility for tumor treatment comprising the steps of removing a tumor biopsy from a patient, explanting the biopsy into tissue culture, introducing a replication-deficient vector into the cells of the biopsy, and assaying for vector replication in the cells. In a preferred embodiment of the invention, the vector is an adenovirus vector.

In a further embodiment of the invention, a method is providing for identifying a tumor, the cells of which contain a factor that allows replication of a replication-deficient vector, or the deficiency of a factor that prevents replication of a replication-deficient vector, by explanting a tumor biopsy, introducing a replication deficient vector into the cells of the biopsy, and quantitating vector DNA replication in the cells. Accordingly, a method is provided for screening tumors for the presence of factors that allow replication of a replication vector, or for a deficiency of a factor that prevents replication of a replication-deficient vector. Such a screen is useful, among other things, for identifying tumors prior to treatment which will be amenable to treatment with a particular vector to be replicated in the tumor cell. In specific embodiments, the tissue to be tested or screened is assayed for tumor-suppressor function, the presence of which prevents vector replication, and the absence of which permits vector replication. In specific disclosed embodiments, the tumor-suppressor functions are the p53 and Rb functions.

Therefore, in a further embodiment of the invention, a method is provided for identifying a tumor with functionally inactivated p53, Rb, or both p53 and Rb, comprising explanting a tumor biopsy, introducing a vector that is unable to replicate in the presence of p53, Rb, or both p53 and Rb, into cells of the biopsy, and quantitating vector DNA replication in the cells. Testing or screening of tissues or tumors, however, is not limited to an assay for viral DNA replication but may also include an assay for virus replication. Potentially, any tissue may be screened for the functions described above by an assay for DNA or virus replication.

In a preferred embodiment, the replication-deficient vector is a DNA tumor viral vector. In preferred embodiments, the DNA tumor viral vector is a vector selected from, but not limited to, the group consisting of herpesvirus, papovavirus, papillomavirus, and hepatitus virus vectors. In a most preferred embodiment, the vector is an adenovirus vector. In a further preferred embodiment, the E1 region, and preferably the E1a region, is deleted from the adenovirus.

In another embodiment of the invention, a cell line is provided containing a virion produced in the cell by replication in the cell of a replication-defective vector, and wherein the replication deficiency is complemented by an endogenous, naturally-occurring cellular function or by the deficiency of an endogenous, naturally-occurring cellular function that inhibits vector replication. In a preferred embodiment, the endogenous, naturally-occurring inhibitor of vector replication that is functionally activated in the producer cell line is the product of a tumor-suppressor gene. In a preferred embodiment, the endogenous, naturally-occurring inhibitor of vector replication that is functionally inactivated in the producer cell line is either p53 or Rb, or both p53 and Rb, or functional equivalents of p53 and/or Rb. The endogenous, naturally-occurring inhibitor of vector replication can preexist in the producer cell. Alternatively, the function can be inactivated by standard recombinant or other methods prior to introduction

or replication of the vector in the cell. In further embodiments, the cell contains multiple copies of the vector or virions.

In a preferred embodiment, the vector is a DNA tumor viral vector and preferably is selected from the group, but is not limited to, herpesvirus, papovavirus, papillomavirus, and hepatitus virus. In a preferred embodiment, the virion is an adenoviral virion and the vector is an adenovirus vector.

In a further preferred embodiment, the E1 region, and preferably the E1a region, is deleted from the adinovirus. In a further preferred embodiment, the cell is functionally inactivated for at least p53 and preferably also for Rb.

In further preferred embodiments, the cell line is a small cell carcinoma cell line. In further preferred embodiments of the invention, the cell line is a hepatoma cell line. Most preferably, the hepatoma cell line is the Hep3B cell line.

In a further preferred embodiment, the vector encodes a heterologous gene product. The heterologous gene product may be a directly toxic drug, or a product that metabolizes a prodrug into a directly toxic drug. Alternatively, the gene product could be a beneficial gene product to be produced in large amounts for further therapeutic application. Further, the producer cell lines are used to produce vectors *per se*, or virions, for further use in gene therapy.

In a further embodiment of the invention, a method is provided for producing a replication-deficient vector or virion, comprising the steps of culturing the cell line described above and recovering the vector or virion from the cell. In preferred embodiments, the cell is a tumor cell. In a still further embodiment, a method is provided for producing replication-deficient DNA tumor virion free of wild-type virions, or DNA tumor viral vector free of wild-type tumor viral vectors, comprising the steps of culturing the producer cell line described above and recovering replication-deficient virions or vectors from the cell.

Brief Description of the Figures

Figure 1: Expression of thymidine kinase activity in AV.TK1 (for construction, see Example 1) infected AV1 replication permissive HuH7 cells.

Figure 2: Representative Southern blot demonstrating cell line specific DNA replication of AV1 vector.

Figure 3: Southern blot demonstrating that cell lines which support replication of AV1 vectors do not contain endogenous E1 genes.

Figure 4: Representative plaque titer demonstrating cell line specific virus replication of AV1 vector.

Figure 5: Complete table summarizing viral cytotoxicity data, DNA replication, and virus production in a variety of tumor cell lines and normal cells.

Figure 6: Table showing the Rb/p53 status of selected cell lines.

Figure 7: Multiplicity of infection vs. cell killing of HuH7.

Figure 8: Ganciclovir mediated bystander effect in HuH7 cells.

Figure 9: Carcinoma cell line specific killing.

Figure 10: Cure rate of nude mice bearing Hep 3B subcutaneous hepatoma treated with AV1.TK1.

Figure 11: p53 dependent ablation of AVI DNA replication in HuH7 cells.

Figure 12: Survival curve for Hep3B tumor-bearing animals treated with various adenovirus vectors.

Detailed Description of the Preferred Embodiments

Definitions

The term "distributing" is intended to mean the spreading of a vector and its attendant heterologous gene (product) (when present on the vector) throughout a target tissue, but especially throughout a tissue mass such as a mass of abnormally proliferating tissue, a non-malignant or malignant tissue. The object of the distribution is to deliver the gene product or the effects of the gene product (as by a bystander effect, for example) to substantially all or a significant number of cells of the target tissue, so as to treat the entire target tissue.

The term "gene product" is intended to mean DNA, RNA, protein, peptides, or viral particles. Thus, the distribution, for the purposes of the invention, is of any of these components.

The term "heterologous gene product" is intended to mean a gene product encoded by a gene not found in the native viral genome.

The terms "replication deficient", "replication minus", or "replication defective" are used interchangeably. These terms are intended to mean a vector or virus having a genetic deficiency resulting in the inability of the virus to replicate in the normal host cell.

The term "abnormally proliferating" is intended to mean a cell having a higher mitotic index than its normally-functioning counterpart, such that there is an abnormal accumulation of such cells.

The term "anti-tumor activity" is intended to mean any activity which inhibits, prevents, or destroys the growth of a tumor.

The term "functional inactivation" is intended to mean a genetic lesion that prevents the normal activity of a gene product. Thus, functional inactivation

could result from a mutation in the gene encoding the gene product. Such a lesion includes insertions, deletions, and base changes. Alternatively, functional inactivation may occur by the abnormal interaction of the normal gene product with one or more other cellular gene products which bind to or otherwise prevent the functional activity of said gene product. Thus, the gene product may be a protein produced from a normal gene but which cannot perform its ordinary and normal function because of an interaction with a second factor.

The term "complemented" is intended to mean the provision of a gene product which provides the function of a gene product inactivated on the viral genome.

The terms "endogenous, naturally-occurring factor" or "endogenous, naturally-occurring function" are intended to mean a gene product encoded by native sequences in the cellular genome. Such factors are to be distinguished from those engineered by recombinant or laboratory methods (as, for example, by the introduction and integration of viral sequences into a cell in the laboratory). I.e., such sequences are a result of natural events and not as a result of human engineering of the cell.

The term "functional equivalent" is intended to mean a gene product arising from a different gene, but having the same biological function; e.g., with respect to p53, such a functional equivalent would interact with viral gene products to achieve the same effect on cells and on viral replication as interaction with E1a does.

The disclosures of all patents, publications (including published patent applications), and database entries referred to in this specification are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication, and database entry were specifically and individually indicated to be incorporated by reference in its entirety.

Treatment

The present invention first provides methods for selectively distributing a polynucleotide in a given tissue *in vivo*, and particularly in tumor tissue, significantly reducing or avoiding distribution in non-target tissue. The polynucleotide is provided in a vector which is selectively distributed in a given tissue, and particularly in a tumor tissue, reducing or avoiding distribution in non-tumor tissue.

The present invention also provides methods for selectively expressing a gene product in a given tissue, and particularly in tumor tissue, avoiding or significantly reducing expression in non-target or non-tumor tissue. The invention provides methods for distribution of the above-mentioned to a greater number of target cells than would be reached using a non-replicating vector. Cells in addition to those first exposed to the polynucleotide, vector, or gene product, are thus potentially reached by the methods.

The methods are specifically directed to the introduction into a target tissue of a normally replication-defective vector, which replicates in the cells of the target tissue. In the target tissue, replication occurs because either the replication deficiency is complemented by a cellular function that allows viral replication, or there is a deficiency in a cellular function that normally prevents or inhibits vector replication. The presence or absence of such functions provides the selectivity that allows the treatment of a specific tissue with minimum effect on the surrounding tissue(s).

In the target tissue, DNA replication alone may occur. Late viral functions that result in packaging of vector DNA into virions may also occur.

The vector may be introduced into the target tissue as naked DNA or by means of encapsidation (as an infectious virus particle or virion). In the latter case, the distribution is accomplished by successive infections of cells in the tissue by the virus such that substantially all or a significant number of the daughter cells are infected.

In a further embodiment of the invention, the vector encodes a heterologous gene product which is expressed from the vector in the tissue cells. The heterologous gene product can be toxic for the cells in the targeted tissue or confer another desired property.

A gene product produced by the vector can be distributed throughout the tissue, because the vector itself is distributed throughout the tissue. Alternatively, although the expression of the gene product may be localized, its effect may be more far-reaching because of a bystander effect or the production of molecules which have long-range effects such as chemokines. The gene product can be RNA, such as antisense RNA or ribozyme, or protein. Examples of toxic products include, but are not limited to, thymidine kinase in conjunction with ganciclovir.

A wide range of toxic effects are possible. Toxic effects can be direct or indirect. Indirect effects may result from the conversion of a prodrug into a directly toxic drug. For example, *Herpes simplex* virus thymidine kinase phosphorylates ganciclovir to produce the nucleotide toxin ganciclovir phosphate. This compound functions as a chain terminator and DNA polymerase inhibitor, and thus is cytotoxic. Another example is the use of cytosine deaminase to convert 5′-fluorocytosine to the anti-cancer drug 5′-fluorouracil. For a discussion of such "suicide" genes, see Blaese, R.M. *et al.*, *Eur. J. Cancer 30A*:1190-1193 (1994).

Direct toxins include, but are not limited to, diphtheria toxin (Brietman *et al.*, *Mol. Cell Biol. 10*:474-479 (1990)), pseudomonas toxin, cytokines (Blankenstein, T., *et al.*, *J. Exp. Med. 173*:1047-1052 (1991), Colombo, M.P., *et al.*, *J. Exp. Med. 173*:889-897 (1991), Leone, A., *et al.*, *Cell 65*:25-35 (1991)), antisense RNAs and ribozymes (Zaia, J.A. *et al.*, *Ann. N.Y. Acad. Sci. 660*:95-106 (1992)), tumor vaccination genes, and DNA encoding for ribozymes.

In accordance with the present invention, the agent which is capable of providing for the inhibition, prevention, or destruction of the growth of the target tissue or tumor cells upon expression of such agent can be a negative selective

marker; i.e., a material which in combination with a chemotherapeutic or interaction agent inhibits, prevents or destroys the growth of the target cells.

Thus, upon introduction to the cells of the negative selective marker, an interaction agent is administered to the host. The interaction agent interacts with the negative selective marker to prevent, inhibit, or destroy the growth of the target cells.

Negative selective markers which may be used include, but are not limited to, thymidine kinase and cytosine deaminase. In one embodiment, the negative selective marker is a viral thymidine kinase selected from the group consisting of *Herpes simplex* virus thymidine kinase, cytomegalovirus thymidine kinase, and varicella-zoster virus thymidine kinase. When viral thymidine kinases are employed, the interaction or chemotherapeutic agent preferably is a nucleoside analogue, for example, one selected from the group consisting of ganciclovir, acyclovir, and 1-2-deoxy-2-fluoro-β-D-arabinofuranosil-5-iodouracil (FIAU). Such interaction agents are utilized efficiently by the viral thymidine kinases as substrates, and such interaction agents thus are incorporated lethally into the DNA of the tumor cells expressing the viral thymidine kinases, thereby resulting in the death of the target cells.

When cytosine deaminase is the negative selective marker, a preferred interaction agent is 5-fluorocytosine. Cytosine deaminase converts 5-fluorocytosine to 5-fluorouracil, which is highly cytotoxic. Thus, the target cells which express the cytosine deaminase gene convert the 5-fluorocytosine to 5-fluorouracil and are killed.

The interaction agent is administered in an amount effective to inhibit, prevent, or destroy the growth of the target cells. For example, the interaction agent is administered in an amount based on body weight and on overall toxicity to a patient. The interaction agent preferably is administered systemically, such as, for example, by intravenous administration, by parenteral administration, by intraperitoneal administration, or by intramuscular administration.

When the vectors of the present invention induce a negative selective marker and are administered to a tissue or tumor *in vivo*, a "bystander effect" may result, i.e., cells which were not originally transduced with the nucleic acid sequence encoding the negative selective marker may be killed upon administration of the interaction agent. Although the scope of the present invention is not intended to be limited by any theoretical reasoning, the transduced cells may be producing a diffusible form of the negative selective marker that either acts extracellularly upon the interaction agent, or is taken up by adjacent, non-target cells, which then become susceptible to the action of the interaction agent. It also is possible that one or both of the negative selective marker and the interaction agent are communicated between target cells.

In one embodiment, the agent which provides for the inhibition, prevention, or destruction of the growth of the tumor cells is a cytokine. In one embodiment, the cytokine is an interleukin. Other cytokines which may be employed include interferons and colony-stimulating factors, such as NM-CSF. Interleukins include, but are not limited to, interleukin-1, interleukin-1β, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, and interleukin-12. In one embodiment, the interleukin is interleukin-2.

In a preferred embodiment of the invention, the target tissue is abnormally proliferating, and preferably tumor tissue. The vector or virus is distributed throughout the tissue or tumor mass.

All tumors are potentially amenable to treatment with the methods of the invention. Tumor types include, but are not limited to hematopoietic, pancreatic, neurologic, hepatic, gastrointestinal tract, endocrine, biliary tract, sino-pulmonary, head and neck, soft tissue sarcoma and carcinoma, dermatologic, reproductive tract, and the like. Preferred tumors for treatment are those with a high mitotic index relative to normal tissue. Preferred tumors are solid tumors, and especially, tumors of the brain, most preferably glioma.

The methods can also be used to target other abnormal cells, for example, cytomegalovirus, Epstein-Barr virus, and human papilloma virus-infected cells. Cytomegalovirus infects a variety of cells, causing many different types of disease (*Virology*, second edition, Fields, B.N. *et al.*, eds., Raven Press Ltd. (1990)). Cytomegalovirus inactivates the p53 gene (Ohno, T., *Science 265*:781-784 (1994); Speir *et al.*, *Science 265*:391-394 (1994)), leading to enhanced proliferation of cells and in some cases detrimental affects. Restenosis is one example of an abnormal cell pathology treatable by the methods of the invention. Following angioplasty, the smooth muscle cells are prone to cytomegalovirus infection or reactivation (Ohno, T., *Science 265*:781-784 (1994); Speir *et al.*, *Science 265*:391-394 (1994)). Thus, the cells proliferate and cause serious blockage of the artery in which angioplasty was performed. These cells could be selectively killed by the methods of the invention, since p53 is inactivated in those cells. In addition, other intraocular lesions develop as a result of cytomegalovirus infection or reactivation. These cells could be selectively eliminated by the methods described herein. Finally, Epstein-Barr virus causes a variety of diseases, including Burkitt's lymphoma. Cells containing this virus, or cells in which this virus has inactivated the p53 gene, can be targeted. Cells containing human papilloma virus (85% of all cervical carcinomas, and several other tumors of the head and neck as well as many papillomas) are also known to eliminate p53, and could be treated with the methods described herein.

In a further embodiment of the invention, the vectors of the present invention can be used to replicate in specific subsets of normal cells. For example, in certain stages of embryogenesis, factors complimenting replication deficiency are known to occur. Vector replication can be exploited in such cell types for the purpose of directed gene expression in these cell types. Therapeutic interventions are achievable in normal tissues if similar conditions favoring DNA replication are produced by the pharmacologic manipulations.

Further, treatment can be *ex vivo*. *Ex vivo* transduction of tumor cells would overcome many of the problems with current viral delivery systems.

Tissue is harvested under sterile conditions, dissociated mechanically and/or enzymatically and cultured under sterile conditions in appropriate media. Vector preparations demonstrated to be free of endotoxins and bacterial contamination are used to transduce cells under sterile conditions *in vitro* using standard protocols. The accessibility of virus to cells in culture is currently superior to *in vivo* injection and permits introduction of vector viral sequences into essentially all cells. Following removal of virus-containing media cells are immediately returned to the patient or are maintained for several days in culture while testing for function or sterility is performed.

For example, patients with hypercholesterolemia have been treated successfully by removing portions of the liver, explanting the hepatocytes in culture, genetically modifying them by exposure to retrovirus, and re-infusing the corrected cells into the liver (Grossman *et al.*, 1994).

Viral transduction also has potential applications in the area of experimental medicine. Transient expression of biological modifiers of immune system function such as IL-2, Ifn-gamma, GM-CSF or the B7 co-stimulatory protein has been proposed as a potential means of inducing anti-tumor responses in cancer patients.

In a further preferred embodiment, the cellular function that prevents virus replication, and in the absence of which function, virus replication occurs, is a tumor-suppressor gene function.

In a further preferred embodiment of the invention, the tumor cells in which the vector or gene product is distributed lack at least functionally active p53 and preferably also lack Rb. The invention also encompasses, however, cells lacking other tumor suppressor functions, such as p107, p130, and p300. For known tumor suppressor genes, see Levine, A.J., *Ann. Rev. Biochem. 62*:623-651 (1993).

Producer Cells

In a further embodiment of the invention, a cell is provided which contains a virion produced in the cell by replication in the cell of a replication-defective vector, wherein the replication deficiency is compensated by an endogenous, naturally-occurring cellular function or replication occurs because of a deficiency in a function that inhibits vector replication. Thus, the invention provides "producer cells" for the efficient and safe production of recombinant replication-defective vectors for further use for gene therapy *in vivo*.

One of the major problems with the currently available producer cells is that such cells contain, in the genome, viral sequences that provide complementing functions for the replicating vector. Because the cell contains such sequences, homologous recombination can occur between the viral sequence in the genome and the viral vector sequences. Such recombination can regenerate recombinant wild-type viruses which contaminate the vector or virus preparation produced in the producer cell. Such contamination is undesirable, as the wild-type viruses or vectors can then replicate in non-target tissue and thereby impair or kill non-target cells. Therefore, one of the primary advantages of the producer cells of the present invention is that they do not contain endogenous viral sequences homologous to sequences found in the vector to be replicated in the cells. The absence of such sequences avoids homologous recombination and the production of wild-type viral recombinants that can affect non-target tissue.

Accordingly, the invention embodies methods for constructing and producing replication-deficient virions in a cell comprising introducing a replication-deficient vector into the cell wherein the genome of the cell is devoid of vector sequences, replicating the vector in the cell wherein the replication-deficiency is compensated by a naturally-occurring endogenous cellular function or deficiency in a function, forming the virion, and purifying the virion from the cell. In preferred embodiments of the invention, the endogenous cellular functi n is provided by tumor-suppressor genes. In more preferable embodiments, the

tumor-suppressor functions are the p53 and Rb tumor-suppressor functions. Preferred vectors are DNA viral vectors, including but not limited to herpesvirus, papillomavirus, hepatitus virus, and papovavirus vectors. In preferred embodiments of the invention, the virion is an adenoviral virion and the vector is an adenoviral vector. In further embodiments of the invention, the cell is a tumor cell which is functionally inactive at least for p53 and preferably also for Rb. In a specific disclosed embodiment, the tumor cell is Hep3b.

In a further preferred embodiment, the vector encodes a heterologous gene product such that the virion also encodes the gene product, and when the vector or virion are used for gene therapy, the therapy is facilitated by expression of the heterologous gene product. Alternatively, the producer cell can be used for the production of a heterologous gene product *per se* encoded by the vector. When the vector replicates in the producer cell, the gene product is expressed from the multiple copies of the gene encoding the gene product. Following expression, the gene product can be purified from the producer cells by conventional lysis procedures, or secreted from the producer cell by appropriate secretion signals linked to the heterologous gene by known methods. The transduction of cells by adenoviral vectors has been described. Transfection of plasmid DNA into cells by calcium phosphate (Hanahan, D., *J. Mol. Biol. 166*:577 (1983)), lipofection (Feigner *et al.*, *PNAS 84*:7413 (1987)), or electroporation (Seed, B., *Nature 329*:840 ( )) has been described. DNA, RNA, and virus purification procedures are described (Graham *et al.*, *J. Gen. Virol. 36*:59-72 (1977).

Preferred producer cells are at least p53 and preferably also Rb-minus. Primary tumors from which cell lines can be derived, or existing cell lines, can be tested for this phenotype. Alternatively, any of the cell lines previously shown to contain Rb and p53 mutations can be used. Examples of primary tumors that could be explanted and developed into producer cells for the vectors of the present invention include small cell lung carcinoma, cervical carcinoma, many tumors of the brain, prostate tumors, colon tumors, lung tumors, sarcomas, and

pancreatic tumors. Cell lines bearing these mutations are also known in the literature and provide candidates for producer cells.

Alternatively, any cell can be chosen whether or not the phenotype is p53 and/or Rb-minus. The cell that is selected on the basis of other desirable characteristics can be manipulated such that the endogenous p53 and/or Rb function is reduced or eliminated. For example, a mutation in either or both of these genes can be introduced by homologous recombination with exogenous mutated DNA. Alternatively, antisense RNA may be used to block p53/Rb expression. Further, functional protein antagonists (that bind and thus inactivate either or both of the proteins) may be introduced into such producer cells as by additional exogenous expression vectors. Such recombinant manipulations for inactivating gene function are well-known to those of ordinary skill in the art.

The ultimate goal for a producer cell line, and particularly an adenoviral producer line, is to produce the highest yield of vector with the least possibility of contamination by wild-type vector. Yield depends upon the number of cells infected. Thus, the more cells that it is possible to grow and infect, the more virus it is possible to generate. Accordingly, candidate cells would have a high growth rate and will grow to a high density. The cell should also have a high amount of viral receptor so that the virus can easily infect the cell. Another characteristic is the quality of the vector produced (i.e., the preparation should not include a high amount of non-infectious viral particles). Accordingly, candidate producer cells would have a low particle-to-plaque-forming-unit ratio. For example, small cell lung carcinoma in general is known to grow well in suspension culture and is one of the fastest growing cancers known (Gazden, A.F. *et al.*, *Cancer Res. 40*:3502 (1980)). In addition, lung cells in general have a high propensity for adenoviral infection (Rosenfeld, M.A. *et al.*, *Cell 68*:143 (1992), and the majority studied to date (Horowitz *et al.*, *PNAS 87*:2775 (1990)) have Rb and p53 mutations. Thus, these cells are a preferred cell type for deriving a producer cell line. Primary explants or the known cell lines can be used.

Further, the p53 and/or Rb in any cell may be mutated or functionally inactivated by homologous recombination with mutated genes (Williams, B.D. *et al.*, *Nature: Genetics 7*:440 (1994)), or by transduction of that cell line with genes that will inactivate these gene products (Yei *et al.*, *Nature 357*:82-85 (1992), Crook *et al.*, *Cell 67*:547 (1991), and DeCaprio *et al.*, *Cell 54*:275 (1988)). Thus, such obtainable cells can serve as producer cells for recombinant replication-deficient vectors, viruses, and gene products.

Diagnostic

The ability to have a method for determining the loss of function of tumor-suppressor genes is important for determining the stage, prognosis, and potential treatment of patients with tumors. In particular, a method is most desirable for determining the loss of function of the tumor-suppressor genes p53 and Rb. The p53 and Rb status is known to play a major role in tumor progression. Further, it is important to know whether current vectors will replicate in a given patient's tumor. If replication is found to be beneficial in therapy, then a screen is provided for those patients who may best respond to this therapy. If it is found to be harmful, then there is a screen for prevention of the treatment of patients who would have an adverse response to the treatment. Currently, the only non-biological assays that are commonly used are expression screening, PCR, and sequencing. These often result in false negatives, are time-consuming, expensive, and yield only information in the best of cases about the status of the genes and not their biological function.

In a further embodiment of the invention, therefore, a method is provided for assaying vector utility in tumor treatment by removing a tumor biopsy from a patient, explanting the biopsy into tissue culture, introducing a replication-deficient vector into the biopsy, and assaying vector replication in the cells of the biopsy. In preferred embodiments of the invention, the vector is an adenovirus

vector. Other vectors, however, may be used in the invention, as discussed herein.

Thus, the invention provides a method for screening a primary tumor for complementation functions that allow vector replication in the presence of tumor-suppressor genes and especially p53 and Rb, or for the absence of functions and especially tumor-suppressor functions which prevent the replication of a virus vector. The determination, therefore, is done by removing a tumor biopsy from the tumor, explanting the biopsy, introducing a replication-deficient vector into cells of the biopsy, and assaying vector replication in the cells. In particular, a method is provided for screening a tumor for functionally inactivated p53 and Rb by means of assaying replication in explanted tumor biopsies. It should be appreciated, however, that such diagnostic methods are not useful only for screening tumors, but for screening any abnormal or normal tissue in which such complementation functions may be present, or in which suppressor functions may be absent.

Introduction of Vectors into Cells

A variety of ways have been developed to introduce vectors into cells in culture, and into cells and tissues of an animal or a human patient. Methods for introducing vectors into mammalian and other animal cells include calcium phosphate transfection, the DEAE-dextran technique, microinjection, liposome mediated techniques, cationic lipid-based techniques, transfection using polybrene, protoplast fusion techniques, electroporation and others. These techniques are well known to those of skill, are described in many readily available publications and have been extensively reviewed. Some of the techniques are reviewed in *Transcription and Translation, A Practical Approach*, Hames, B.D. and Higgins, S.J., eds., IRL Press, Oxf rd (1984), herein incorporated by reference in its entirety, and *Molecular Cloning*, Second Edition,

Maniatis *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989), herein incorporated by reference in its entirety.

Several of these techniques have been used to introduce vectors into tissues and cells in animals and human patients. Chief among these have been systemic administration and direct injection into sites *in situ*. Depending on the route of administration and the vector, the techniques have been used to introduce naked DNA, DNA complexed with cationic lipid, viral vectors and vector producer cell lines into normal and abnormal cells and tissues, generally by direct injection into a targeted site.

The aforementioned techniques for introducing polynucleotide, viral and other vectors into cells in culture, in animals and in patients can be used to develop, test and produce, as well as use vectors in accordance with the invention. For instance, cells containing a vector introduced by these methods can be used for producing the vector. In addition, cells containing a vector can be used as producer-cells and introduced into cells or tissues of an animal to produce the vector *in situ*.

Assay of DNA and Viral Replication

Replication of a polynucleotide, viral or other vector can be assayed by well-known techniques. Assays for replication of a vector in a cell generally involve detecting a polynucleotide, virions or infective virus. A variety of well-known methods that can be used for this purpose involve determining the amount of a labelled substrate incorporated into a polynucleotide during a given period in a cell.

When replication involves a DNA polynucleotide, ^{3}H-thymidine often is used as the labelled substrate. In this case, the amount of replication is determined by separating DNA of the vector from the bulk of cellular DNA and measuring the amount of tritium incorporate specifically into vector DNA.

Replication of a polynucleotide vector also may be detected by lysing or permeating cells to release the polynucleotide, then isolating the polynucleotide and quantitating directly the DNA or RNA that is recovered. Polynucleotide replication also may be detected by quantitative PCR using primers that are specific for the assay polynucleotide.

Virions may be assayed by EM counting techniques well known to the art, by isolating the virions and determining protein and nucleic acid content, and by labelling viral genomic polynucleotides or virion proteins and determining the amount of virion from the amount of polynucleotide or protein.

It is well known that virions may not all be viable and where infectivity is important, infectious titer may be determined by cytopathic effect or plaque assay.

Any of these well-known techniques, among others, can be employed to assay replication of a vector in a cell or tissue in accordance with the invention. It will be appreciated that different techniques will be better suited to some vectors than others and to some cells or tissues than others.

Vectors

The preferred vectors of the present invention are adenoviral vectors. Adenovirus is preferred, among other things, because it rarely integrates into the host genome, and, therefore, if used to express a beneficial heterologous gene, it will not cause host genetic mutation. In a preferred embodiment of the invention, an adenovirus vector is deleted in the E1 region.

The adenovirus E1b 55 kDa protein functions both in the disruption of p53-mediated apoptosis (for review, Moran, E., *FASEB J.* 7:880-885 (1933)) and in cooperative interactions with Ad E4 34 kDa protein to facilitate accumulation of late viral mRNAs (Ornelles & Shenk, *J. Virol.* 65:425-439 (1991). E1-deleted adenovirus DNA replication which occurs in p53 negative (dysfunctional (e.g., inactivated by other cellular functions) or deleted p53) cells is only rarely

accompanied by production of infectious virus (Figure 5). This phenomenon may be related to the absence of E1b 55 kDa-E4 34 kDa interaction.

Add1312 (Jones & Shenk, *Cell 17*:683-689 (1979)) is deleted for E1a but retains a functional E1b transcription unit as well as the wild-type E4 region. Mutagenesis of the E1b region in Add1312, followed by selection on cells known to be deleted or dysfunctional for the genes encoding p53 (such as C-33, a cervical carcinoma cell line) should allow selection of viral E1b 55 kDa mutants retaining essential interactions with E4 34 kDa but lacking p53 interaction capabilities.

Therefore, in a further preferred embodiment, the methods use a vector that retains its ability to replicate DNA in p53 defective cells and furthermore displays increased virion production by virtue of the more efficient processing and transport of late viral RNAs, the step at which E1a-E1b deleted vector virion production in p53 negative cells appears blocked. Vectors of this class are capable of spreading among p53 negative (deleted and/or dysfunctional) cells as replicating virions, though replication of either vector viral DNA or virions would remain impossible or greatly reduced in cells with normal p53 function.

In one embodiment, both E1a and E1b are deleted. In an alternative embodiment, only the E1a region is deleted from the E1 region; the E1b region remains intact. In the latter case, a cellular complementation function may allow virus replication to proceed by means of a factor that compensates for E1a deficiency and the viral E1b region allows cellular processing and transport functions to be utilized for late virus product and virus packaging and release. This E1b function is particularly desirable in cells that are p53-minus (i.e., that do not contain a functionally active p53). Further, either one or both of the E1b functions may be included or deleted from the E1a vector.

In a specific disclosed embodiment, a vector for use in the methods of the invention is the AV1.TK1 vector. For a detailed description of vect r construction, see Example 1 herein. The generati n of AV1.TK1 is identical to the generation of AV1.LacZ4, except that the thymidine kinase gene is inserted

in place of the LacZ gene. This (as well as AV1.LacZ4) vector is constructed by operably linking the *Herpes simplex* virus type 1 thymidine kinase gene (or the β-gal gene containing a nuclear localizing signal (GenBank J01636)) to the Rous Sarcoma Virus promotor on pAVS6, an adenoviral shuttle plasmid containing the left ITR of Ad5 and another region homologous to Ad5 (Trapnell, B. *et al.*, *Adv. Drug Deliv. Rev 12*:185-189 (1994)). After linearization of the plasmid, it is cotransfected into 293 cells with a replication-incompetent subgenomic fragment of Ad5 that has the E3 region deleted (Addl327) (Yei, S. *et al.*, *Human Gene Therapy 5*:731-744 (1994)). Upon homologous recombination, a recombinant virus is generated. Virus is then plaque-purified on 293 cells, and purified as previously described (Graham, F.L. *et al.*, *J. Gen. Virol. 36*:59-72 (1977)). Addl312 is an E1a deletion mutant generated as previously described (Shenk *et al.*, *Cold Spring Harbor Symp. Quant. Biol. 44*:367-375 ( )).

In alternative embodiments, adenovirus vectors are provided with deletions in any of the other genes essential for replication, such as E2-E4.

In further alternative embodiments, the vector is derived from another DNA tumor virus. Such viruses generally include, but are not limited to the herpesviruses (such as Epstein-Barr virus, cytomegalovirus, *Herpes zoster*, and *Herpes simplex*), papillomaviruses, papovaviruses (such as polyoma and SV40), and hepatitis viruses. The alternative viruses preferably are selected from the group of viruses that inactivate Rb or p53. All serotypes are included. The common property is a viral gene that inactivates a cellular function(s) that prevents DNA replication or promotes apoptosis. Most preferably, the viral gene(s) perform a substantially identical function to the E1a and E1b genes found in adenovirus and its serotypes. Examples of genes include, but are not limited to, the E6 and E7 regions of human papilloma virus, 16 and 18 T antigen of SV40, and CMV immediate early genes. Any of the viruses which infect cells, and particularly human cells, could have either specific portions or the entire E1 region counterpart deleted and thus be used in all the methods described herein, in which methods an adenovirus deletion mutant could be used.

The invention further embodies the use of plasmids and vectors having only the essential regions of adenovirus needed for replication with either E1a, E1b 19kDa gene, or E1b 55kDa gene, or some combination thereof, deleted. Such a plasmid, lacking any structural genes, would be able to undergo DNA replication as the E1-deleted adenovirus vectors do.

The vectors described herein can be constructed using standard molecular biological techniques. Standard techniques for the construction of such vectors are well-known to those of ordinary skill in the art, and can be found in references such as Sambrook *et al.*, in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York (1989), or any of the myriad of laboratory manuals on recombinant DNA technology that are widely available. A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments and can be readily determined by the skilled artisan.

The adenoviral vector may be a modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted or mutated so that a functional viral product is not produced (Shenk *et al.*, *Curr. Top. Microbiol. Immunol. 111(3)*:1-39 (1984)).

In one embodiment, the vector comprises an adenoviral 5′ ITR; an adenoviral 3′ ITR; an adenoviral encapsidation signal; a DNA sequence containing a heterologous gene. The vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, but is not necessarily free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter.

In still another embodiment, the gene in the E2a region that encodes the 72 kilodalton binding protein is mutated to produce a temperature sensitive protein that is active at 32°C, the temperature at which viral particles are produced but is inactive at 37°C, the temperature of the animal or human host. This temperature sensitive mutant is described in Ensinger *et al.*, *J. Virology 10*:328-339 (1972); Van der Vliet *et al.*, *J. Virology 15*:348-354 (1975); and

Friefeld *et al.*, *Virology 124*:380-389 (1983); Englehardt *et al.*, *Proc. Nat. Acad. Sci. 91*:6196-6200 (1994); Yang *et al.*, *Nature: Genetics 7*:362-369 (1994).

Such a vector, in a preferred embodiment, is constructed first by constructing, according to standard techniques, a shuttle plasmid which contains, beginning at the 5′ end, the "critical left end elements," which include an adenoviral 5′ ITR, an adenoviral encapsidation signal, and an E1a enhancer sequence; a promoter (which may be an adenoviral promoter or a foreign promoter); a tripartite leader sequence, a multiple cloning site (which may be as herein described); a poly A signal; and a DNA segment which corresponds to a segment of the adenoviral genome. Such DNA segment serves as a substrate for homologous recombination with a modified or mutated adenovirus. The plasmid may also include a selectable marker and an origin of replication. The origin of replication may be a bacterial origin of replication. Representative examples of such shuttle plasmids include pAVS6, as discussed herein and see Trapnell, B. *et al.*, *Adv. Drug Deliv. Rev 12*:185-189 (1994). A desired DNA sequence containing a heterologous gene may then be inserted into the multiple cloning site to produce a plasmid vector.

This construct then is used to produce an adenoviral vector. Homologous recombination then is effected with a modified or mutated adenovirus in which at least the majority of the E1 and E3 adenoviral DNA sequences have been deleted. Such homologous recombination may be effected through co-transfection of the plasmid vector and the modified adenovirus into a helper cell line by $CaPO_4$ precipitation.

Through such homologous recombination, a vector is formed which includes an adenoviral 5′ ITR, an adenoviral encapsidation signal; an E1a enhancer sequence; a promoter; a tripartite leader sequence; a DNA sequence containing the heterologous gene; a poly A signal; adenoviral DNA free of at least the majority of the E1 and E3 adenoviral DNA sequences; and an adenoviral 3′ ITR. This vector may then be transfected into a helper cell line for viral replication and to generate infectious viral particles. Transfections may take

place by electroporation, calcium phosphate precipitation, microinjection, or through proteoliposomes.

The vector may include a multiple cloning site to facilitate the insertion of DNA sequence(s) containing the heterologous gene into the cloning vector. In general, the multiple cloning site includes "rare" restriction enzyme sites; i.e., sites which are found in eukaryotic genes at a frequency of from about one in every 10,000 to about one in every 100,000 base pairs. An appropriate vector is thus formed by cutting the cloning vector by standard techniques at appropriate restriction sites in the multiple cloning site, and then ligating the DNA sequence containing the heterologous gene into the cloning vector.

The DNA sequence encoding the heterologous gene product is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the Rous Sarcoma Virus promoter; inducible promoters, such as the MMTV promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoE promoter; and the ApoAI promoter. It is to be understood, however, the scope of the present invention is not limited to specific foreign genes or promoters.

In one embodiment, the adenovirus may be constructed by using a yeast artificial chromosome (or YAC) containing an adenoviral genome according to the method described in Ketner, *et al.*, *Proc. Nat. Acad. Sci. 91*:6186-6190 (1994), in conjunction with the teachings contained herein. In this embodiment, the adenovirus yeast artificial chromosome is produced by homologous recombination *in vivo* between adenoviral DNA and yeast artificial chromosome plasmid vectors carrying segments of the adenoviral left and right genomic termini. A DNA sequence containing the heterologous gene then may be cloned into the adenoviral DNA. The modified adenoviral genome then is excised from the adenovirus yeast artificial chr mosome in order to be used to generate infectious adenoviral particles.

The infectious viral particles may then be administered *in vivo* to a host. The host may be an animal host, including mammalian, non-human primate, and human hosts.

The viral particles may be administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier (for example, a saline solution), or a solid carrier, such as, for example, microcarrier beads.

Having thus described herein the invention in general terms, the following examples are presented to illustrate the invention.

Example 1

Construction of pAVS6

The adenoviral construction shuttle plasmid pAVS6 was constructed in several steps using standard cloning techniques including polymerase chain reaction based cloning techniques. First, the 2913 bp BglII, HindIII fragment was removed from Add1327 and inserted as a blunt fragment into the XhoI site of pBluescript II KS (Stratagene, La Jolla, CA). Add1327 (Thimmappaya *et al.*, *Cell 31*:543-551 (1983), incorporated herein by reference) is identical to Adenovirus 5 except that an XbaI fragment including bases 28591 to 30474 (or map units 78.5 to 84.7) of the Adenovirus 5 genome, and which is located in the E3 region, has been deleted. The complete Adenovirus 5 genome is registered as Genbank accession #M73260, incorporated herein by reference, and the virus is available from the American Type Culture Collection, Rockville, Maryland, U.S.A. under accession number VR-5.

Add1327 was constructed by routine methods from Adenovirus 5 (Ad5). The method is outlined briefly as follows an previously described by Jones and Shenk, *Cell 13*:181-188, (1978). Ad5 DNA is isolated by proteolytic digestion

of the virion and partially cleaved with XbaI restriction endonuclease. The XbaI fragments are then reassembled by ligation as a mixture of fragments. This results in some ligated genomes with a sequence similar to Ad5, except excluding sequences 28591 bp to 30474 bp. This DNA is then transfected into suitable cells (e.g. KB cells, HeLa cells, 293 cells) and overlaid with soft agar to allow plaque formation. Individual plaques are then isolated, amplified, and screened for the absence of the 1878 bp E3 region XbaI fragment.

The orientation of this fragment was such that the BglII site was nearest the T7 RNA polymerase site of pKSII and the HindIII site was nearest the T3 RNA polymerase site of Pbluescript II KS. This plasmid was designated pHR.

Second, the ITR, encapsidation signal, Rous Sarcoma Virus promoter, the adenoviral tripartite leader (TPL) sequence and linking sequences were assembled as a block using PCR amplification. The ITR and encapsidation signal (sequences 1-392 of Add1327 [identical to sequences from Ad5, Genbank accession #M73260], incorporated herein by reference) were amplified (amplification 1) together from Add1327 using primers containing NotI or AscI restriction sites. The Rous Sarcoma Virus LTR promoter was amplified (amplification 2) from the plasmid pRC/Rous sarcoma virus (sequences 209 to 605; Invitrogen, San Diego, CA) using primers containing an AscI site and an SfiI site. DNA products from amplifications 1 and 2 were joined using the "overlap" PCR method (amplification 3) (Horton *et al.*, *Biotechniques* 8:528-535 (1990)) with only the NotI primer and the SfiI primer. Complementarily between the AscI containing end of each initial DNA amplification product from reactions 1 and 2 allowed joining of these two pieces during amplification. Next the TPL was amplified (amplification 4) (sequences 6049 to 9730 of Add1327 [identical to similar sequences from Ad5, Genbank accession #M73260]) from cDNA made from mRNA isolated from 293 cells (ATCC accession No. CRL 1573) infected for 16 hrs. with Add1327 using primers containing SfiI and XbaI sites respectively. DNA fragments from amplification reactions 3 and 4 were then

joined using PCR (amplification 5) with the NotI and XbaI primers, thus creating the complete gene block.

Third, the ITR-encapsidation signal-TPL fragment was then purified, cleaved with NotI and XbaI and inserted into the NotI, XbaI cleaved PHR plasmid. This plasmid was designated pAvS6A and the orientation was such that the NotI site of the fragment was next to the T7 RNA polymerase site.

Fourth, the SV40 early polyA signal was removed from SV40 DNA as an HpaI-BamHI fragment, treated with T4 DNA polymerase and inserted into the SalI site of the plasmid pAVS6a to create pAVS6.

Construction of Av1LacZ4

The recombinant, replication-deficient adenoviral vector Av1LacZ4, which expresses a nuclear-targetable B-galactosidase enzyme, was constructed in two steps. First, a transcriptional unit consisting of DNA encoding amino acids 1 through 4 of the SV40 T-antigen followed by DNA encoding amino acids 127 through 147 of the SV40 T-antigen (containing the nuclear targeting peptide Pro-Lys-Lys-Lys-Arg-Lys-Val), followed by DNA encoding amino acids 6 through 1021 of *E. coli* B-galactosidase, was constructed using routine cloning and PCR techniques and placed into the EcoRV site of pAVS6 to yield pAVS6-nlacZ.

The infectious, replication-deficient, AvlLacZ4 was assembled in 293 cells by homologous recombination. To accomplish this, plasmid pAVS6-Nlacz was linearized by cleavage with KpnI. Genomic adenoviral DNA was isolated from purified Add1327 viruses by Hirt extraction, cleaved with ClaI, and the large (approximately 35 kb) fragment was isolated by agarose gel electrophoresis and purified. The ClaI fragment was used as the backbone for all first generation adenoviral vectors, and the vectors derived from it are known as AV1.

Five micrograms of linearized plasmid DNA (pAVS6n-LacZ) and 2.5 μg of the large ClaI fragment of Add1327 then were mixed and co-transfected into

a dish of 293 cells by the calcium phosphate precipitation method. After 16 hours, the cells were overlaid with a 1:1 mixture of 2% Sea Plaque agar and 2x medium and incubated in a humidified, 37°C, 5% CO_2/air environment until plaques appeared (approximately one to two weeks). Plaques were selected and intracellular vector was released into the medium by three cycles of freezing and thawing. The lysate was cleared of cellular debris by centrifugation. The plaque (in 300 µl) was used for a first round of infection of 293 cells, vector release, and clarification as follows:

One 35 mm dish of 293 cell was infected with 100 µl of plaque lysate plus 400 µl of IMEM-2 (IMEM plus 2% FBS, 2mM glutamine (Bio Whittaker 046764)) plus 1.5 ml of IMEM-10 (Improved minimal essential medium (Eagle's) with 2x glutamine plus 10% vol./vol. fetal bovine serum plus 2mM glutamine plus (Bio Whittaker 08063A) and incubated at 37°C for approximately three days until the cytopathic effect, a rounded appearance and "grapelike" clusters, was observed. Cells and supernatant were collected and designated as CVL-A. AvlLacZ4 vector (a schematic of the construction of which is shown in Figure 10) was released by three cycles of freezing and thawing of the CVL-A. Then, a 60 mm dish of 293 cells was infected with 0.5 ml of the CVL-A plus 3 ml of IMEM-10 and incubated for approximately three days as above. Cells and supernatant from this infection then were processed by three freeze/thaw cycles in the same manner. Av1LacZ4 also is described in Yei *et al.*, *Human Gene Therapy* 5:731-744 (1994); Trapnell, *Advanced Drug Delivery Rev. 12*:185-199 (1993), and Smith *et al.*, *Nature: Genetics* 5:397-402 (1993), which are incorporated herein by reference.

Example 2

Cytopathic effect of E1-deleted adenovirus in E1⁻ cell lines

A survey of tumor cell lines was undertaken to identify potential candidates for *in vitro* and *in vivo* models of tumor killing using adenovirus-thymidine kinase constructs in conjunction with ganciclovir. A number of cell lines were tested, including human glioma cell lines U87, U373, and U118, a hepatocellular carcinoma cell line Hep 3B, and Y79, a human retinoblastoma cell line. The most prominent effect occurred with Hep 3B. The cell line proved to be exquisitely sensitive to adenovirus exposure, showing significant cytopathic effects, even at low levels of input virus.

To test the hypothesis that viral replication was causing the cytopathic effects, cells were exposed to varying amounts of virus and observed for the development of cytopathic effects.

Hep3B cells were plated at 1 million cells per well of 6-well dishes in RPMI 1640 media containing 10% fetal calf serum. Transductions of Hep3B cells were performed by adding AV.lacZ4 virus (6×10^{10} plaque forming units (pfu)/ml) at ratios of 5000, 1600, 530, 175, 55, 18, 6, 2, 0.2, and 0.06 pfu/cell. Uninfected cells served as a control. Cells were observed for cytopathic effects for a period of 15 days. Cells not showing cytopathic effects at day 8 were trypsinized and re-plated at 5×10^5 cells per well and followed for an additional 7 days. Cytopathic effects were vacuole formation in the cytoplasm progressing to displacement of the nucleus, nuclear densities, crenulations of the cell membrane, and cell rounding with loss of adherence and/or cell-cell contacts. The results are shown in Table 1.

Table 1

Results:	Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Input virus (MOI)	5000	-	+	L	X												
	1600	-	+	L	X												
	530	-	+	+	L	X											
	175	-	+	+	L	X											
	55	-	-	+	+	L	X										
	20	-	-	-	+	+	L	X									
	6.5	-	-	-	-	-			L	X							
	2	-	-	-	-	-											L
	0.6	-	-	-	-	-											L
	0.2	-	-	-	-	-											L
	0.06	-	-	-	-	-											-
	uninfected	-	-	-	-	-											-

- = no cytopathic effect noted
+ = cytopathic effects present as defined above
L = lysis/disintegration of cells
X = all cells released from monolayer, presumed dead

The results show morphological changes consistent with virus replication. Such changes previously had only been known to occur in cells infected with wild type adenovirus or in cells constructed to specifically trans-complement mutant adenoviruses (293).

The absence of the E1 region genes in the mutant AV.lacZ should have rendered this virus incompetent for replication. Nonetheless, evidence of replication was present. The results indicate that the tumor cells either contain viral DNA sequences which complement the E1 deficiency or that the cells have accumulated sufficient mutations to create an environment which would permit virus replication in the absence of E1 genes or E1 homologs (for example, HPV 16 or 18 E6/E7, CMV immediate early genes, SV40 T-antigen).

As the function of the adenovirus E1 genes include specific inactivation of cellular p53 and Rb gene products, the results suggest that a deficiency in the function of these cellular genes allows replication.

Example 3

Expression of thymidine kinase activity in AV.TK1 infected AV1 replication permissive HuH7 cells

In order to determine the quantity of activity of a gene product expressed from an AV1 vector in cells which support replication of AV1 vectors the human hepatoma cell line HuH7 was infected with AV1.TK1. This vector expresses thymidine kinase activity from the Rous sarcoma virus promoter in the vector. This vector was chosen since previous work (Culver *et al.*, *Science 256*:1550-1557) has demonstrated that thymidine kinase actively positively correlates with cell-killing by ganciclovir and the bystander effect (the ability of cells expressing thymidine kinase in the presence of ganciclovir to kill neighboring cells not expressing thymidine kinase). HuH 7 cells were chosen because as demonstrated herein, these cells support replication of AV1 vectors.

For each thymidine kinase activity measurement, 1 x 10^7 cells were infected with either AV1.LacZ4 at an MOI of 100 as a negative control for thymidine kinase activity or with AV1.TK1 at MOIs of 10 and 100. Infections were performed as previously described (Graham, F.L. *et al., J. Gen. Virol. 36*:56-72 (1977)). NIH 3T3 thymidine kinase-cells were also harvested and used as a negative control. PA317/G1TK1SvNa#7 is a cell line that produces high amounts of thymidine kinase. The line was previously optimized to express thymidine kinase from multiple endogenous copies of a retrovirus thymidine kinase gene. This line was used as a positive control for thymidine kinase activity. Equivalent amounts of protein were assayed from the samples for thymidine kinase activity as previously described. Thymidine kinase activity was measured two days following infection.

The results are shown in Figure 1. No thymidine kinase activity was found in NIH 3T3 thymidine kinase-cells alone or in HuH 7 cells infected with AV1.LacZ4 at an MOI of 100. High levels of thymidine kinase activity were present in PA317/G1TK1SVNa#7 cells. HuH 7 cells infected with an AV1.TK1 at an MOI of 10 showed a significant amount of thymidine kinase activity. However, HuH 7 cells infected with AV1.TK1 at an MOI of 100 showed approximately 3 fold higher thymidine kinase activity than the optimized producer cell line PA317/G1TK1SVNa#7 and 30 fold higher activity than HuH 7 cells infected with AV1.TK1. This result suggests that the high thymidine kinase activity in AV1.TK1 infected cells is due to higher gene dosage from replication of AV1. This conclusion is based on the observation that thymidine kinase activity in HuH 7 cells appears to be exponential with increasing MOI: greater than 30 fold higher activity was found in cells infected with an MOI of 100 vs an MOI of 10. If thymidine kinase activity linearly increased with increasing MOI, a 10 fold increase would be expected, as has been demonstrated for other cell lines which may not support replication of AV1 vectors (*Cancer Gene Therapy 1*:107-112 (1994)).

The levels of thymidine kinase activity were also shown to correlate with the amount of protein by a quantitative Western analysis using a monoclonal antibody specific to thymidine kinase. That DNA replication of adenovirus leads to a dramatically enhanced gene dosage has been demonstrated previously utilizing a luciferase reporter gene (Mittal, S.K. *et al.*, *Virus Res. 28(1)*:67-90 (1994)).

Representative Southern blot demonstrating cell line specific DNA replication of AV1 vector

The possibility was investigated that E1 deleted adenoviral vectors could replicate in a variety of tumor cell lines. Cell lines were infected with the AV1.LacZ4 vector. Adenoviral DNA accumulation over time indicates DNA replication.

1 x 10^5 cells of each of the various cell lines were plated into 6 well dishes. Semi-confluent monolayers were formed within 24h. 24h later, cells were infected with either AV1.LacZ4 E1 deleted vector, or as a positive control for replication, wild-type virus and Add1327, both at an MOI of 10. Infections were performed as described for Fig. 1. Samples were harvested at 4h, two days, and six days, after infection by standard trypsinization of adherent cells immediately following a 2 ml wash of the monolayer by PBS. Cells were collected by centrifugation at 3000 x g to recover the residual cell pellet. The cell pellet was resuspended once with 1 ml PBS and respun. Pellets were frozen on dry ice and stored at -70 until isolation of DNA. DNA was isolated from frozen cell pellets as previously described (*Molecular Cloning: A Laboratory Manual*, second edition, Sambrook, J. *et al.*, eds., Cold Spring Harbor Laboratory Press (1989)). One third of the total DNA isolated from each well was digested to completion by Hind III and electrophoresed on a 1% agarose gel. Gels were transferred onto Nytran, prehybridized, and hybridized, as previously described (*Molecular Cloning: A Laboratory Manual*, second edition, Sambrook, J. *et al.*,

eds., Cold Spring Harbor Laboratory Press (1989)). Probes were a 400 bp PCR generated and purified probe specific for the E2b region of adenovirus (which should hybridize to a specific Hind III restriction fragment of adenovirus), and a 200 bp PCR generated and purified probe specific for the E1a region (which should hybridize to a specific Hind III fragment of adenovirus). The E2b probe should hybridize to both AV1 DNA and Add1327 DNA, whereas the E1a probe should only hybridize to the AV1 DNA since this region is deleted in AV1 vectors.

The results are shown in Figure 2. The results demonstrate that the wild-type viral DNA was substantially increased in every cell line tested. The AV1.LacZ4 vector replicated in 293 cells. This result is not unexpected since 293 cells harbor and express the E1 region deleted in most currently used adenoviral vectors (including the AV1 vectors herein). However, DNA replication of the AV1 vector was observed in Hep3B cells, at a level approaching that found in 293 cells. DNA replication was also observed in HuH 7 cells and, on a much longer exposure, A549 cells. Although there was no apparent replication at an M01 of 10 of the AV1.LacZ4 vector in SW480 cells, preliminary experimental evidence has been obtained that replication of AV1 DNA does occur at the slightly higher MOI of 30 in SW480 cells.

To further conclude that what was observed with the E2b probe was replication of AV1 DNA, and not replication of a small amount of contaminating wild-type virus, the blot was stripped by boiling twice in 0.1 M EDTA (*Molecular Cloning: A Laboratory Manual*, second edition, Sambrook, J. *et al.*, eds., Cold Spring Harbor Laboratory Press (1989)) and reprobed with the E1a probe. The E1a probe hybridized only to the expected Hind III restriction fragment and only in cells infected with wild-type virus. This confirms that the AV1.DNA is replicated in the tumor cell lines. These results indicate that replication of AV1 occurs in cell lines that are not known to harbor the E1 genes.

Demonstration that cell lines which support replication of AV1 vectors do not contain endogenous E1 genes

Although it was exceedingly unlikely that human cell lines would have an integrated adenoviral sequence (*Virology*, second edition, Fields, B.N. *et al.*, eds., Raven Press Ltd. (1990)), to determine unambiguously that there were no integrated E1 sequences in Hep3B and SW480, DNA from these lines was analyzed by Southern blot.

1 x 10^7 cells from actively growing cultures from each cell line were harvested by centrifugation at 3000 xg. DNA was harvested by an automated system per the manufacturer's instructions (Genepure Extractor Model 341, Perkin Elmer/Applied Biosystems Division, Foster City, CA). 10 µg of DNA, digested with Hind III, was electrophoresed on a 1% gel, and transferred to nitrocellulose. Hybridization conditions and the preparation of the E1 probe were as described for Figure 2. Another probe for the entire adenovirus of Add1327 (which is identical to Ad 5 except that the E3 region is deleted) was made by random priming of purified viral DNA. The results are shown in Figure 3. Although both probes hybridized to 293 DNA as expected, neither probe hybridized to DNA isolated from either Hep 3B or SW480 cells. This indicates that the factor complementing the E1 defect in the AV1 vector is not related to genes present on the adenovirus backbone.

Representative plaque titer demonstrating cell line specific virus replication of AV1 vector

Since high levels of AV1 vector DNA replication were observed in a variety of tumor cell lines, the inventors determined if infectious virus also could be produced from tumor cell lines supporting DNA replication. Each cell line was plated at a density of 1 x 10^5 cells in a six well plate and allowed to adhere for 24h before infection. Cells from 293 cells (positive control), Hep3B, SW480,

and A549 were infected at an MOI of 10 with AV1.LacZ4. Plates were washed at 4h with PBS and the infection was allowed to proceed for 6d before preparing a CVL as described (Graham, F.L. *et al.*, *J. Gen. Virol. 36*:56-72 (1977)). Plaque titers on 293 cells (Graham, F.L. *et al.*, *J. Gen. Virol. 36*:56-72 (1977)) were performed on each CVL to determine the amount of infectious virus produced from the cells over the 6d period.

The results are shown in Figure 4. No detectable virus was found in any of the 4h time points on any of the cell lines. This result was anticipated because the virus cannot replicate in this short time period. 293 cells produced approximately 4500 pfus/starting cell as expected. Although A549 and SW480 produced no detectable virus (although they supported a low level of DNA replication), a large amount of virus was produced in Hep3B cells. These cells produced nearly as much infectious virus as 293 cells without optimizing the conditions (1500 pfus/starting cell). The results demonstrate that Hep3B cells, and potentially other tumor cells, are fully capable of complementing the E1 deletion in AV1 vectors.

Since the AV1 vector does not contain the gene which expresses E1b 55 kDa polypeptide (which has the function of transporting viral MRNA into the cytoplasm for translation) (Ornelles, D.A. and Shenk, T., *J. Virology 65*:424-439 (1991)), the question was asked whether the presence of this protein might enhance virus production because of higher translation of viral encoded proteins. Add1312 (Shenk, T. *et al.*, *Cold Spring Harbor Symp. Quant. Biol. 44*:367-375), which is deleted in E1a but contains E1b, was utilized. When Hep3B cells were infected with Add1312 at an MOI of 10, approximately six-fold more virus was produced compared to virus produced with AV1.LacZ4.

Complete table summarizing viral cytotoxicity data, DNA replication, and virus production in a variety of tumor cell lines and normal cells

Other cell lines were examined for each of the three properties of AV1 infected cells (cytotoxicity, DNA replication, and virus production) to determine the extent of tumor cell lines capable of permitting replication of an AV1 vector. Normal cells were also examined.

Each assay was performed as described above. The results are shown in Figure 5. + refers to arbitrary grading system for simplicity; for DNA replication, ++++ = maximum observed effect (compared to effect observed with Hep 3B cells); +++ = 50-75%; ++ = 25-50%; + = 10-25%; — = none detectable. For cytotoxicity (Cyto) only ++++, +++, and ++ were used. Less than ++ is a - in this assay. NT = not tested. IP = In progress. pub = published.

The data demonstrate that AV1 infection leads to slowed growth (cytotoxicity) in many tumor cell lines, but not in normal fibroblasts, hepatocytes, or lung cells. When DNA replication was measured, it was found that DNA replication occurred in all cells demonstrating any significant CPE (as well as in several others). However, no DNA replication was observed at this MOI in normal fibroblasts (Figure 5), a normal lung line (Yei, S. *et al.*, *Hum. Gene Ther.* *5*:731-744 (1994)), or in primary hepatocytes (Kozarsky, K. *et al.*, *Somat. Cell Mol. Genet. 19(5)*:449-58 (1993)). Of the cell lines tested, only Hep3B cells were capable of producing infectious virus at the sensitivity examined.

Table showing the p53/Rb genotypes of cell lines

The function of the E1a and E1b region in adenovirus is to inactivate the cellular Rb and p53 gene products, respectively. Since the E1 deleted adenoviral vector was replicating in a variety of tumor cells, it was possible that the tumor cell lines in which DNA replication was observed harbored functionally inactivated Rb and p53, and could thus complement the E1 defect.

All tumor cell lines which supported DNA replication (shown in bold in Figure 6) were found in the literature to contain either mutated or null p53 genes or a functionally inactivated p53 protein (SIHA/HeLa). All lines which did not support DNA replication had a wild type p53 gene. Thus, the ability of AV1 vectors to replicate in tumor cell lines correlates with the p53 status of the line. Although an Rb mutation alone did not allow DNA replication of the AV1 vector (DU-145), many of the cell lines harboring a functionally inactivated or mutated p53 gene were also shown to contain a functionally inactivated or mutated Rb gene.

MOI vs cell killing of HuH7

An experiment was performed to examine if replication could enhance the ability of a vector carrying a suicide gene. The gene is the *Herpes simplex* type 1 thymidine kinase gene. The gene product is known to render tumor cells sensitive to the nucleotide analogue of ganciclovir. The human hepatoma cell line HuH7 was infected. The results are shown in Figure 7. The cell line, which supports DNA replication of the vector, showed greater than 90% kill rate by a relatively MOI of 5 in the presence of 50 μm ganciclovir. In addition, when a MOI of 50 was used, greater than 80% of the cells were killed in a two-day time period in the absence of ganciclovir, and 100% of the cells were killed in the presence of ganciclovir. In similar studies utilizing other cells which may not support DNA replication, much higher MOIs were necessary to achieve a similar degree of tumor cell killing by ganciclovir, and in the absence of ganciclovir, no tumor cell killing was observed (Smythe, W.R. *et al.*, *Cancer Res. 54(8)*:2055-2059 (1994)).

Ganciclovir mediated bystander effect in HuH7 cells

Cells bearing herpesvirus thymidine kinase-expressing vectors are known to cause neighboring cells not expressing the thymidine kinase gene to become sensitive to ganciclovir by a "bystander effect." The inventors have obtained data suggesting that at least *in vitro* (and presumably *in vivo*), the higher the thymidine kinase expression in tumor cells, the more neighboring tumor cells can be killed. Therefore, the question of whether a cell line which supports replication could cause a high bystander effect was examined.

HuH7 cells infected with a MOI of 50 were mixed with increasing quantities of uninfected cells (holding the total number of cells constant) and were treated with 50 μM ganciclovir. The results are shown in Figure 8. If no bystander effect occurred, a linear response between 100% infected cells (all killed) and 0% infected cells (all living) would have been expected. However, it was observed that when only 1% of the cells were infected, nearly 80% of the cells were killed. This dramatic bystander effect may be due to the enhanced gene dosage expected from DNA replication of the vector. The bystander effect should significantly contribute to tumor cell killing, since it is difficult to achieve high transduction rates of *in situ* tumors with any vector (Blaese, R.M. *et al.*, *Eur. J. Cancer 30A*:1191-1193 (1994)). These results, combined with the results described above, suggest that replication significantly enhances the ability of E1-deleted vectors to specifically kill tumor cells.

Carcinoma cell line specific killing

In order to determine if infection of AV1 vectors caused a cytotoxic effect in certain infected tumor cells, a crystal violet staining assay was utilized (Yamamoto *et al.*, in *Practical Methods in Chemical Immunology*, Volume 9: *Investigation of Cell Mediated Immunity*, Yoshida, T., ed., Churchill Livingstone, Edinburgh, New York (1985), pp. 127-134), in which the level of crystal violet

uptake is proportional to the amount of adherent live cells. 1 x 10^4 cells of each tumor cell line was plated into each of three wells in a 96 well plate. 24h after plating, cells were infected with either diluent, Add1327, or AV1.LacZ4, both at an MOI of 10. Samples were assayed for crystal violet uptake in triplicate, 2d, 6d, and 9d following infection.

The results are shown in Figure 9. As expected, cells treated with diluent in each of the four cell lines tested showed in increase in crystal violet staining over time, indicating cell growth. 293 cells were rapidly killed by either the wild-type or AV1 vector, as expected, since this cell line supports replication of the virus. Add1327 killed either A549 Hep3B or HuH7 cells within the six-day period. However, the AV1 vector killed greater than 90% of either the Hep3B cells or HuH7 cells within the six-day period. This result was unexpected, since E1-deleted adenoviral vectors have not caused cytotoxic effects at this MOI in a wide variety of normal tissues (Kozarsky, K. *et al.*, *Somat. Cell Mol. Genet. 19(5)*:449-458 (1993); Yei, S. *et al.*, *Hum. Gene Ther. 5*:731-744 (1994)) and other cell lines (Smythe, W.R. *et al.*, *Cancer Res. 54(8)*:2055-2059 (1994)). The AV1 vector had a reproducible effect on cell growth of the A549 cells as well.

The results suggest that: (1) replication of the AV1 vector occurs in a cell-specific manner, since cytotoxicity and cytopathic effects are indicative of replication, and (2) replication was tumor-cell-line-specific at these MOIs. An absence of cytotoxic effects of this vector in primary human hepatocytes even up to a MOI of 100 has been demonstrated by the inventors and also by others. Furthermore, a number of tumor cell lines and normal cells have been screened, with the result that cytotoxicity was found only in cells which have either mutated or inactivated p53 (see Figures 5 and 6).

Cure rate of nude mice bearing Hep 3B subcutaneous hepatoma treated with AV1.TK1

Since Hep 3B cells were shown to support both virion and DNA replication, the ability of AV1.TK1 vector was tested to either kill or slow growth of a preestablished Hep 3B tumor in nude mice. Hep 3B tumors were established by injecting 1 x 10^7 cells subcutaneously on the flank of a nude mouse. Tumors were allowed to grow until they reached an average size of 50 mm^3. Tumors were then injected with 2 x 10^9 pfus of either AV1.null or AV1.TK1 or diluent alone. Virus was injected in a total volume of 100 ul with two injections through the outer skin. One half of each group was treated with gancyclovir (ganciclovir), at 300 mg/kg/day.

The results are shown in Figure 10. All animals treated with AV1.TK1 and ganciclovir showed complete regression of tumors with 50% remaining tumor free after 30 days. No adverse effect was observed in mice treated this way. These results show that a vector which specifically replicates in tumor cells can be used for cancer therapy.

p53-dependent ablation of AV1 DNA replication in HuH7 cells

To directly demonstrate that reintroduction of wild-type p53 in cells which were devoid of wild-type p53 and which supported replication of the AV1 vector, p53 was introduced into HuH7 cells and cells were assayed for replication. An AV1.p53 vector was constructed and produced in 293 cells as described herein. Since this vector expresses wild-type p53, cells infected with this virus should not be capable of replicating the vector as they do AV1.LacZ4.

The results are shown in Figure 11. Cells infected with Addl327, or AV1.LacZ4 showed an increase in viral DNA over time, indicating replication. However, cells infected with AV1.p53 showed no increase in DNA over time. These results demonstrate that reintroduction of p53 prevents replication.

Survival curve for Hep3B tumor-bearing animals treated with various adenoviral vectors

Athymic nude mice with Hep3B tumors averaging 95 mm^3 were treated by direct intratumoral injection of AV1.lacZ4 (E1a/E1b-deleted virus expressing an irrelevant transgene), AV1.TK1 (E1a/E1b-deleted virus expressing a transgene which converts gancyclovir (GCV) to a toxic metabolite), or dl327 (E1a/E1b-containing adenovirus which replicates in a wild-type fashion in human cells *in vitro*). Survival curves are based upon the sacrifice of animals when tumor volume exceeded 2,000 mm^3 or animals were found dead.

The ability of E1a/E1b-deleted adenovirus to ablate tumors *in vivo* is demonstrated in Figure 12. In the context of Hep3B cells, which support the replication of E1a/E1b-deleted adenovirus at a level similar to wild-type replication, tumor ablation can occur as a direct consequence of infection with dl327 (wild type). Importantly, AV1.lacZ4 ablates the tumor as well as dl327, and does so as effectively as an E1-deleted virus containing a suicide gene (AV1.TK1 plus gancyclovir).

Complementation of viral replication in tumor cells

Results obtained herein using Hep3B cells and an AVI vector show evidence for a complementation function for defective replication functions in the vector. In the experiments described in the exemplary material herein, it is shown that of all the tumor cell lines screened, many of which were p53- and/or Rb-, only Hep3B cells were capable of making infectious viral particles. Most of the Rb- and/or p53- tumor cells replicated the DNA of the adenoviral genome, but only Hep3B cells produced infectious virus. See, for example, Figure 5.

Thus, the data support involvement of complementing factors for full infectious virus production. The data also show that CPE and cytotoxicity can occur in the complete absence of any infectious virus production.

Cells which complement the replication deficiencies of E1a/E1b-deleted adenoviruses must permit several biochemical events to proceed. These events include blockage of apoptosis and the accumulation of the transcription factor E2F (E2F exerts much of the positive transcriptional control over the activity of the E4 and E2 promoters and subsequently over the progression of adenoviral replication from the DNA synthetic phase to the phase of capsid production and assembly). Naturally-occurring cellular factors which completely overcome the block to infectious virus production by E1a-deleted viral vectors include nflL-6 (Spergel *et al., Proc. Natl. Acad. Sci. (USA) 88*(15):6472-6476 (1991)). The need for mutations in, or inactivation of, the pRb family (and possibly p53 as well) of gene products by E1a is merely a means of increasing the concentration of active E2F. Further, the active form of this factor is found in all mitotically-active cells (including tumor cells) as part of cell cycle progression. E1b-mediated blockage of apoptosis may also be dispensable, as other naturally-occurring cellular peptides can block apoptosis as well (e.g., proteins from the bcl-2 locus).

However, cells permissive for virion production by E1a/E1b deleted vectors must also possess additional factors. These factors are necessary to transcomplement functions formally produced by interactions between E1a/E1b gene products and other viral regulatory proteins (e.g., the interaction of E1b with E4 gene products, necessary for the preferential processing and transport of mRNAs encoding the viral late gene products (Ornelles *et al., J. Virol. 65*(1):425-439 (1991)). Most cells lacking either p53 or pRb function do not efficiently produce infectious virions (see Figs. 5 and 6). In the absence of transcomplementing activities which supplant the need for protein interactions between E1a/E1b-encoded proteins and products of the other adenovirus genes, cells may support viral DNA replication but can not efficiently support infectious virion producti n (Fig. 5). Hep3B cells, which support vigorous viral replication, must possess one or more factors that will transcomplement the defect arising from the absent E1a/E1b interactions with other viral gene products. It has been shown (Martuza *et al., Science 252*:854-856 (1991)) that a thymidine kinase

defect in a *Herpesvirus* can be overcome in tumor cells, which produce significantly more TK than normal cells do. This complementation allows the virus to replicate in the tumor cells.

What Is Claimed Is:

1. A method for distributing a polynucleotide in a tissue *in vivo* comprising introducing a replication-deficient vector containing said polynucleotide into said tissue, wherein said vector replicates in the cells of said tissue, wherein said replication deficiency is complemented by one or more endogenous, naturally-occurring factors that allow the replication of said replication-deficient vector in said cell, or wherein an endogenous, naturally-occurring inhibitor of vector replication is functionally inactive in said cell, thereby allowing vector replication.

2. The method of claim 1 wherein said tissue comprises abnormally proliferating cells.

3. The method of claim 2 wherein said tissue is tumor tissue.

4. The method of claim 1 wherein said vector is a DNA tumor viral vector.

5. The method of claim 4, wherein said DNA tumor viral vector is selected from the group consisting of herpesvirus, papovavirus, papillomavirus, and hepatitis virus vectors.

6. The method of claim 1 wherein said vector is an adenoviral vector.

7. The method of claim 6 wherein the E1 region is deleted from said adenovirus.

8. The method of claim 1 wherein said vector encodes a heterologous gene product and wherein said vector expresses said heterologous gene product in the cells of said tissue.

9. The method of claim 8 wherein said heterologous gene product provides anti-tumor activity in the cells of said tissue.

10. The method of claim 1 wherein said vector is introduced into said tissue by virus infection.

11. The method of claim 10 wherein said virus is an adenovirus.

12. The method of claim 3 wherein said tumor is functionally inactive for either p53, Rb, or both p53 and Rb.

13. A cell containing a replication-deficient virion produced in said cell by replication in said cell of a replication-deficient vector, wherein said replication-deficiency is complemented by one or more endogenous naturally occurring factors in said cell that allow the replication of said replication-deficient vector in said cell, or wherein one or more endogenous, naturally-occurring inhibitors of vector replication are functionally inactive in said cell, thereby allowing vector replication in said cell, and wherein said cell is not a HeLa cell.

14. The cell of claim 13, wherein said cell is a small cell carcinoma cell.

15. The cell of claim 13, wherein said endogenous, naturally-occurring inhibitor of vector replication that is functionally inactivated is either p53 or Rb, or both p53 and Rb, or functional equivalents of said p53 and/or Rb.

16. The cell of claim 13 wherein said cell is a hepatoma cell.

17. The cell of claim 13 wherein said hepatoma cell is Hep3B.

18. The cell of claim 13 wherein said cell is a lung carcinoma cell.

19. A method of producing replication-deficient DNA tumor virions free of wild-type virions comprising the steps of culturing the cell of claim 13 and recovering said replication-deficient virions from said cell.

20. The cell of claim 13, wherein said DNA tumor viral vector is selected from the group consisting of herpesvirus, papovavirus, papillomavirus, and hepatitis virus.

21. The cell of claim 13 wherein said virion is an adenoviral virion and said vector is an adenoviral vector.

22. The cell of claim 13 wherein the E1 region is deleted from said adenovirus.

23. The cell of claim 13 wherein said cell is functionally inactive for either p53 or Rb or both p53 and Rb.

24. The cell of claim 13 wherein said cell is a hepatoma cell.

25. The cell of claim 13 wherein said vector encodes a heterologous gene product.

26. A method for producing a replication-deficient virion comprising the steps of culturing the cell of claim 13 and recovering said virion from said cell.

27. The method of claim 26 wherein said cell is a tumor cell.

28. The method of claim 26 wherein said vector is introduced into said cell by transduction.

29. The method of claim 26 wherein said vector is introduced by transfection.

30. A method for identifying a tumor, the cells of which contain one or more factors that allow replication of a replication-deficient vector which are deficient in one or more functionally active factors that prevent replication of a replication-deficient vector, by explanting a tumor biopsy, introducing said replication-deficient vector into the cells of said biopsy, and quantitating vector DNA replication in said cells.

31. A method for identifying a tumor for functionally-inactivated p53, Rb, or both p53 and Rb, comprising explanting a tumor biopsy, introducing a vector that is unable to replicate in the presence of p53, Rb, or both p53 and Rb, into cells of said biopsy, and quantitating vector DNA replication in said cells.

32. A method of treating a tumor in a mammal *in vivo* comprising delivering an effective amount of an infectious replication-deficient DNA tumor virus vector to said tumor, wherein said DNA tumor virus vector replicates in the cells of said tumor and not in the non-tumor cells of said mammal, and wherein the replication of said DNA tumor virus causes a cytotoxic effect in said tumor cells.

33. The method of claim 32, wherein said DNA tumor virus is an adenovirus.

34. The method of claim 32, wherein said cytotoxic effect is caused by the lysis of said tumor cell.

35. The method of claim 32, wherein said cytotoxic effect is caused by a gene product encoded by the DNA of said DNA tumor virus.

36. The method of claim 35, wherein said gene product is either toxic to said tumor cells, is metabolized in said cells into a product that is toxic to said tumor cells, or catalyzes the production of an agent that is toxic to said tumor cells.

37. The method of claim 35, wherein said gene product provokes a cytotoxic response by the immune system of said mammal.

38. The method of claim 32, further comprising the step of determining whether said tumor cells contain one or more factors that allow said DNA tumor virus vector to replicate in said tumor cells or are functionally inactive for one or more factors that inhibit the replication of said DNA tumor virus vector, said step occurring prior to delivering said DNA tumor virus vector to said tumor.

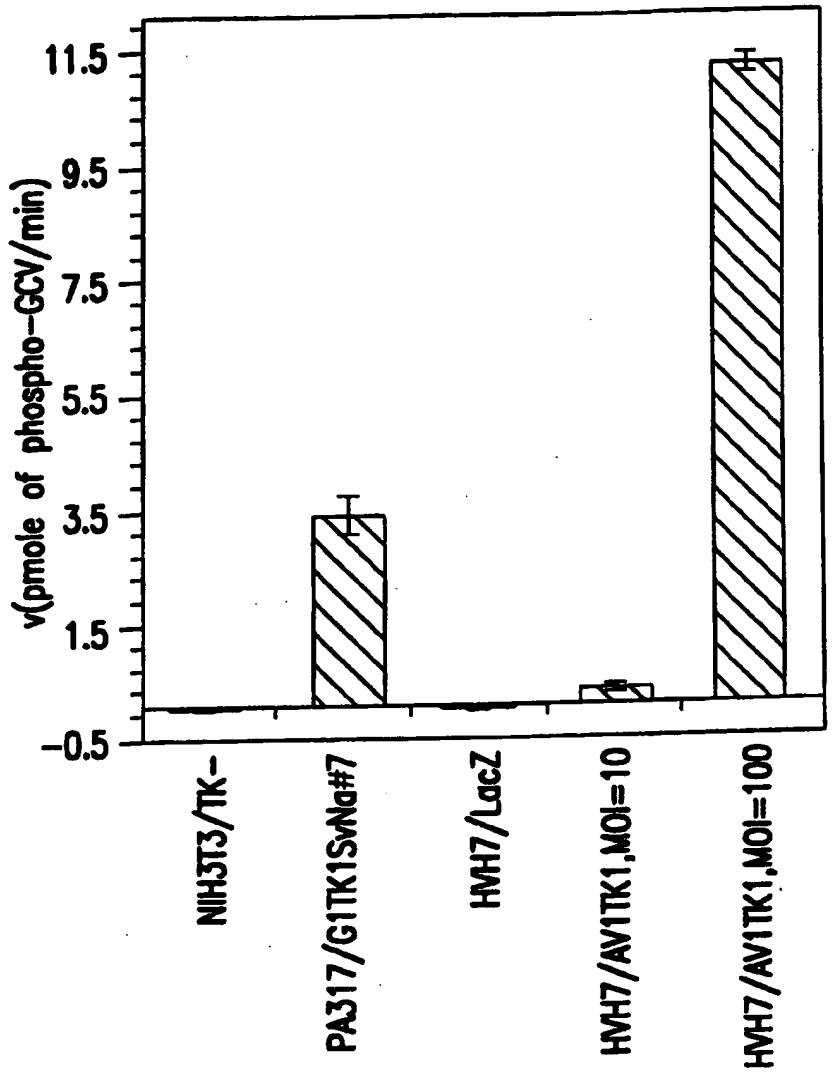
v(pmole of phospho-GCV/min)
11.5
9.5
7.5
5.5
3.5
1.5
-0.5
NIH3T3/TK-
PA317/G1TK1SvNa#7
HVH7/LacZ
HVH7/AV1TK1,MOI=10
HVH7/AV1TK1,MOI=100

FIG.1

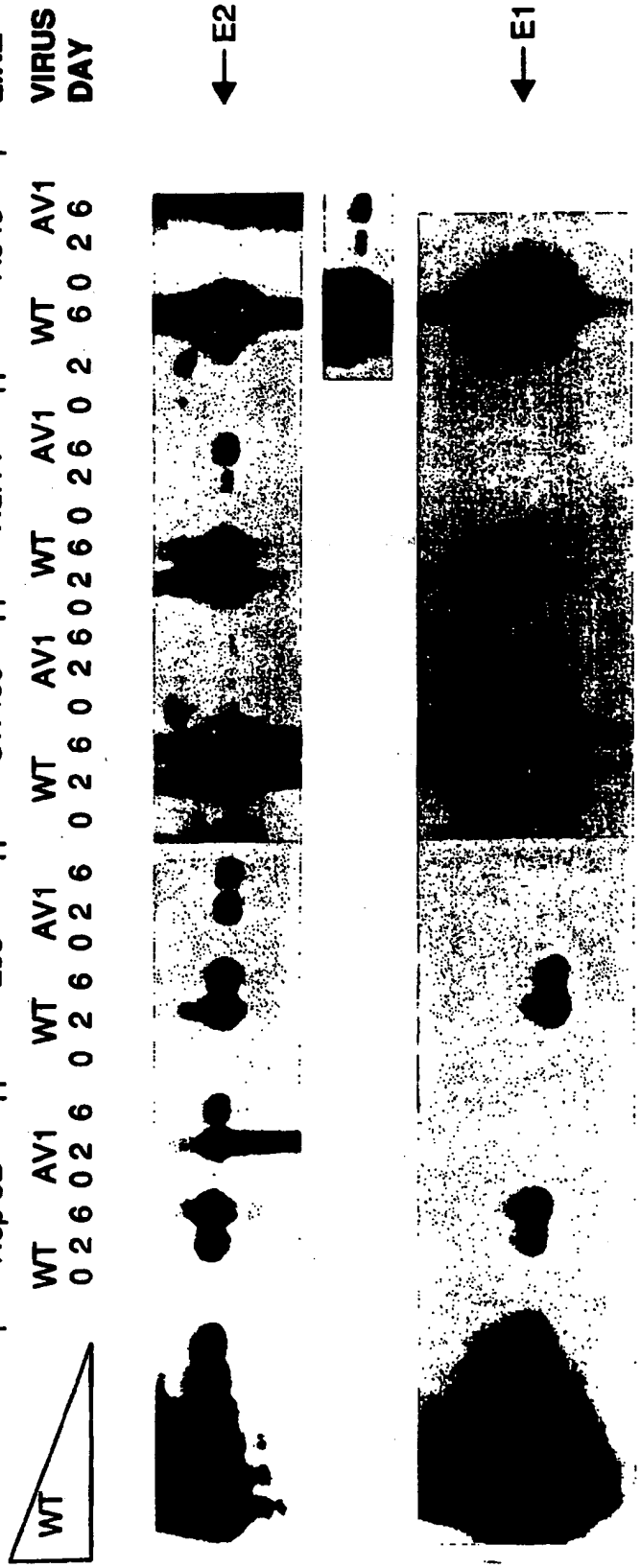
Cell Line Specific Replication
of AV1 Vector
CELL LINE
VIRUS
DAY
Hep 3B
293
SW480
HuH 7
A549
WT AV1 WT AV1 WT AV1 WT AV1 WT AV1
0 2 6 0 2 6 0 2 6 0 2 6 0 2 6 0 2 6 0 2 6 0 2 6 0 2 6 0 2 6
E2
E1
WT

FIG.2

FIG.3

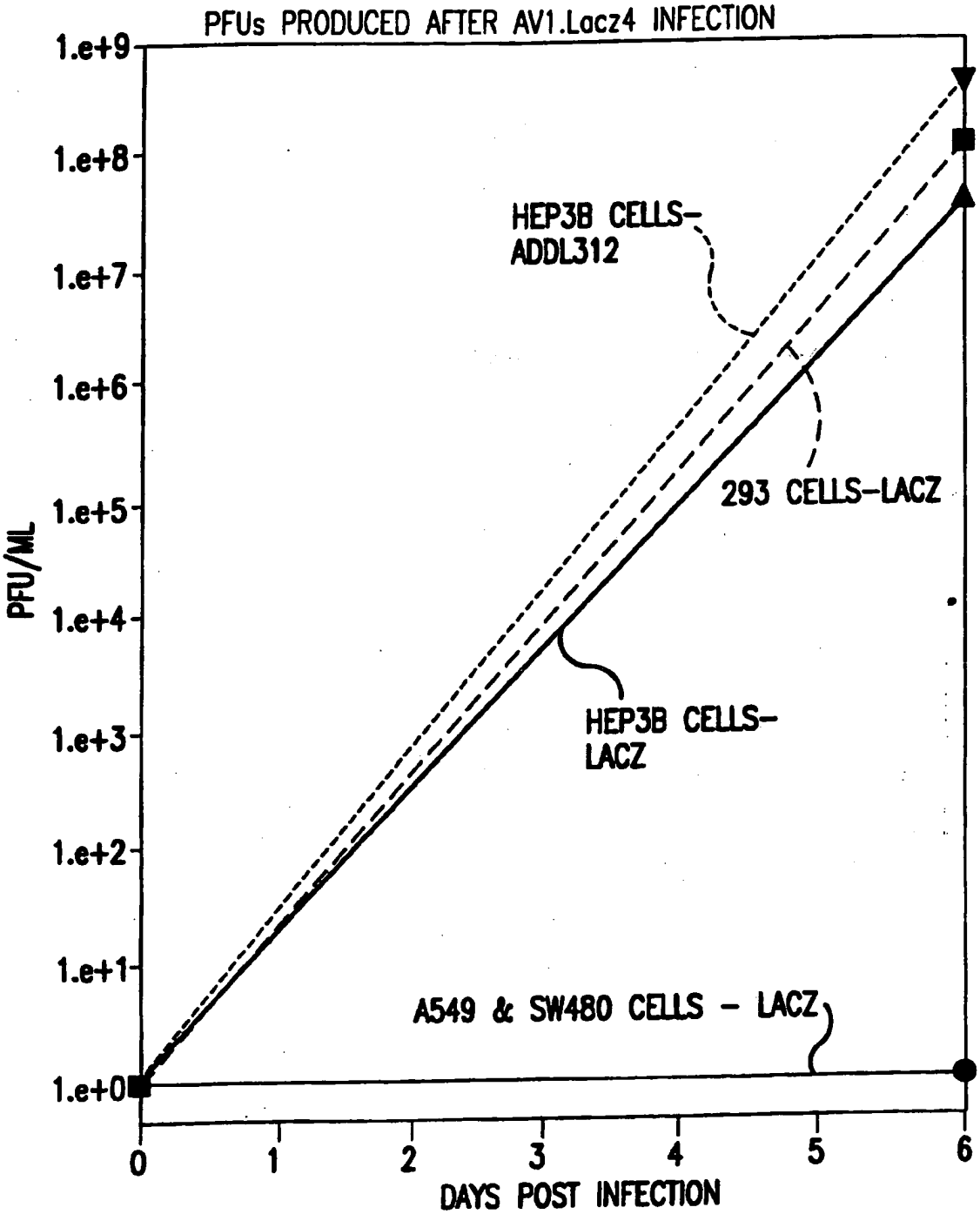
PFUs PRODUCED AFTER AV1.Lacz4 INFECTION
1.e+9
1.e+8
1.e+7
1.e+6
1.e+5
1.e+4
1.e+3
1.e+2
1.e+1
1.e+0
PFU/ML
HEP3B CELLS-
ADDL312
293 CELLS-LACZ
HEP3B CELLS-
LACZ
A549 & SW480 CELLS - LACZ
0
1
2
3
4
5
6
DAYS POST INFECTION

FIG.4

CARCINOMA SPECIFIC VIRUS REPLICATION BY AV1.LacZ4

CELL LINE		CYTO	DNA REP	VIRUS REP
HEPATOMA	HuH 7	+++	+++	-
	Hep 3B	++++	++++	++++
	HLF	++	NT	NT
	SK Hep 1	++	NT	NT
	Hep G2	-	++(Pub)\NT	NT
GLIOMA	U118	IP	++	-
	U373	+++	IP	-
	U87	IP	IP	-
	E2.BT	IP	IP	-
COLON	SW480	-	-	-
CERVICAL	SIHA	+++	IP	IP
	Hela	++	++(pub)	-
BREAST	MDA-468	+++	IP	IP
LUNG(NSCLC)	H460	-	NT	NT
	H358	-	NT	NT
	A549	-	+	-
PROSTATE	DU-145	-	-	-
	PC-3	-	+++	-
OVARIAN	SKOV3	-	NT	NT
	OVCAR3	-	NT	NT
SARCOMA	SAOS-2	-	IP	IP
RETINOBLASTOMA	Y79	+++	IP	IP
NORMAL	FIBROBLAST	-	-	-*
	HEPATOCYTE	-	IP-(pub)	IP
	LUNG	-	-(pub)	IP

FIG.5

Rb^-P53^-	Rb^+P53^-	Rb^-P53^+	Rb^+P53^+
Hep 3B	HuH 7	DU-145	LUNG
U118	PC-3		FIBROBLAST
Y79	A549		HEPATOCYTE
MD-468	SW480		
SIHA\Hela			

FIG.6

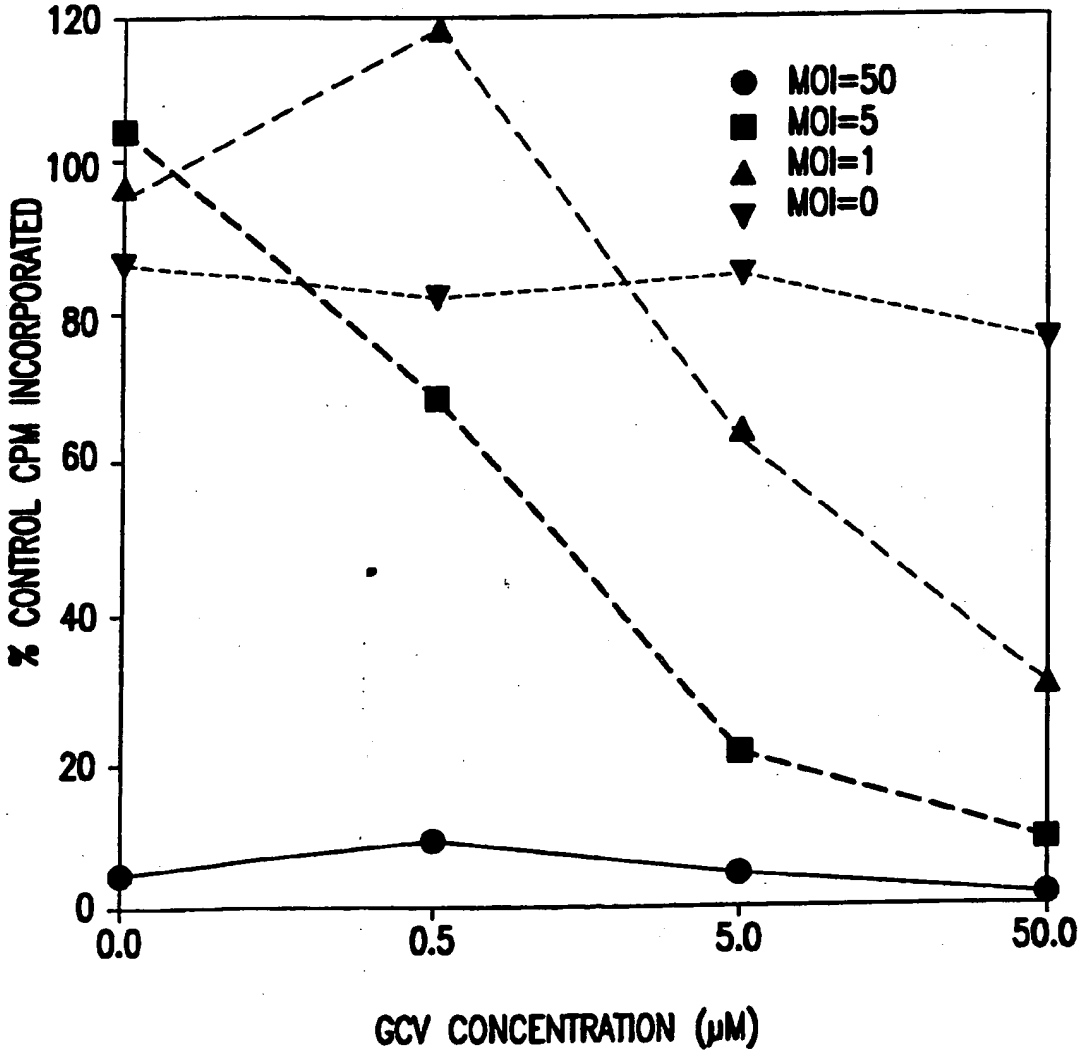
120
100
80
60
40
20
0
% CONTROL CPM INCORPORATED
MOI=50
MOI=5
MOI=1
MOI=0
0.0
0.5
5.0
50.0
GCV CONCENTRATION (μM)

FIG.7

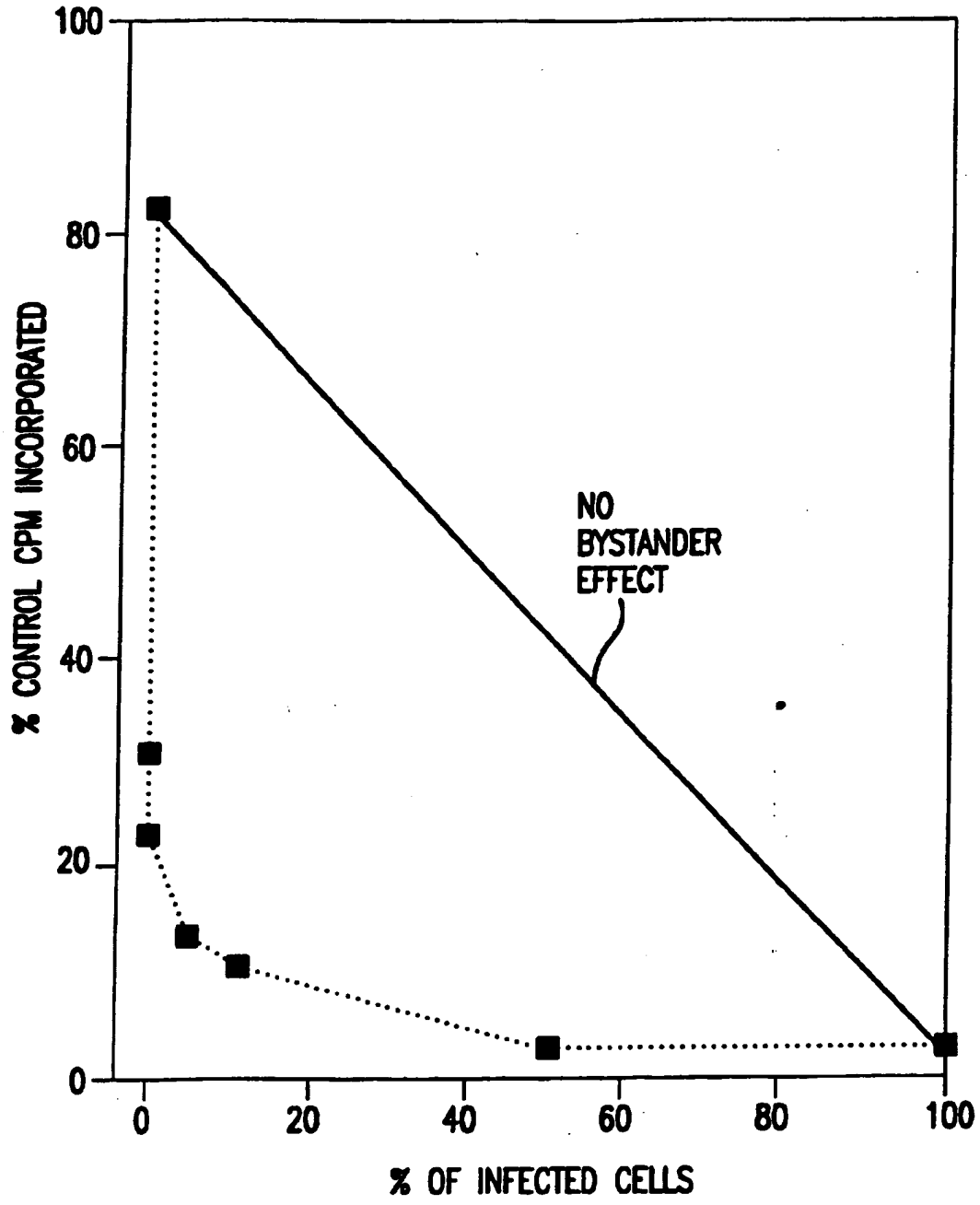
100
80
60
40
20
0
% CONTROL CPM INCORPORATED
NO
BYSTANDER
EFFECT
0
20
40
60
80
100
% OF INFECTED CELLS

FIG.8

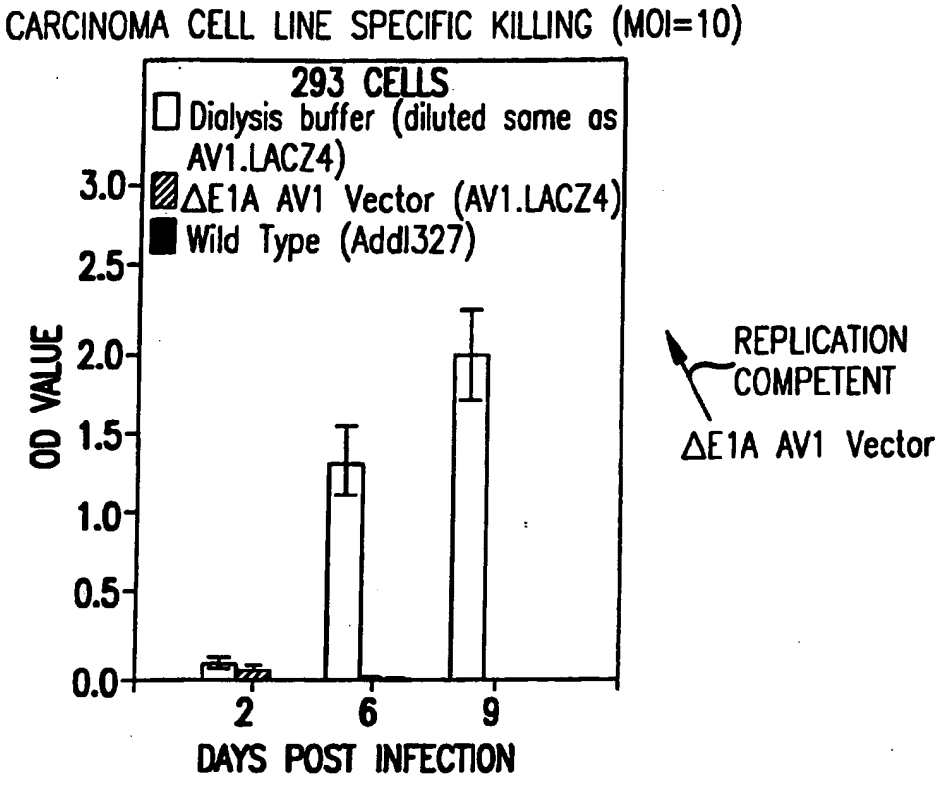
CARCINOMA CELL LINE SPECIFIC KILLING (MOI=10)
293 CELLS
Dialysis buffer (diluted same as AV1.LACZ4)
ΔE1A AV1 Vector (AV1.LACZ4)
Wild Type (Addl327)
OD VALUE
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
6
9
DAYS POST INFECTION
REPLICATION COMPETENT
ΔE1A AV1 Vector

FIG.9A

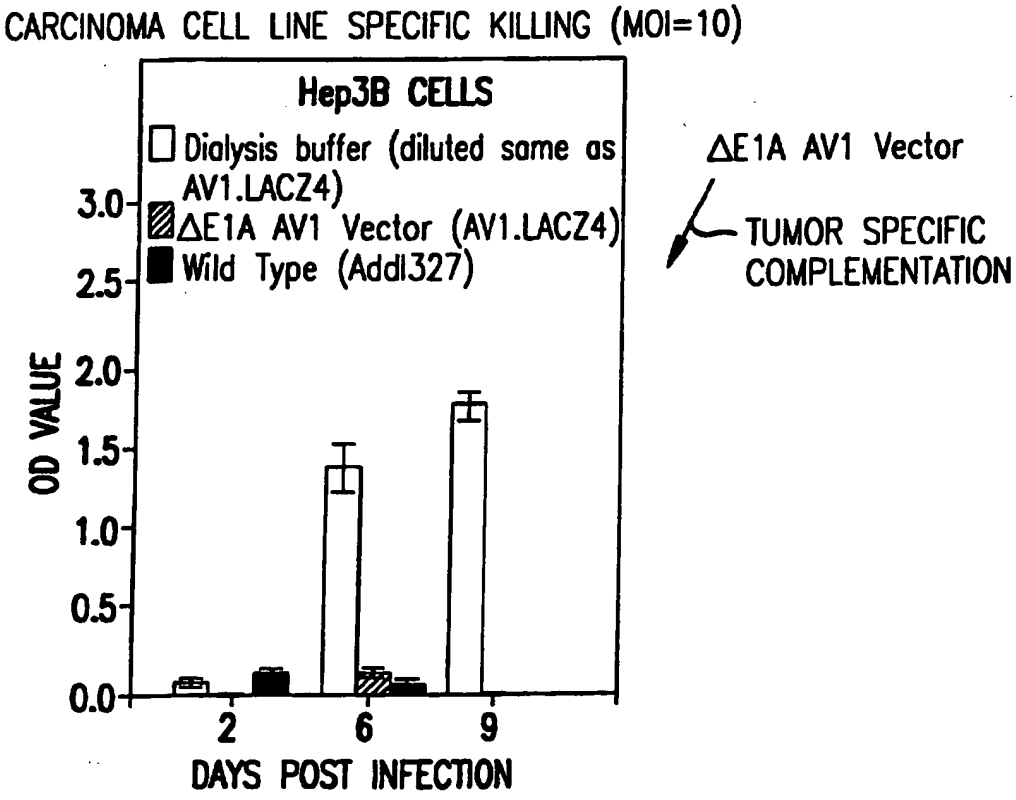
CARCINOMA CELL LINE SPECIFIC KILLING (MOI=10)
Hep3B CELLS
Dialysis buffer (diluted same as AV1.LACZ4)
ΔE1A AV1 Vector (AV1.LACZ4)
Wild Type (Addl327)
OD VALUE
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
6
9
DAYS POST INFECTION
ΔE1A AV1 Vector
TUMOR SPECIFIC COMPLEMENTATION

FIG.9B

CARCINOMA CELL LINE SPECIFIC KILLING (MOI=10)

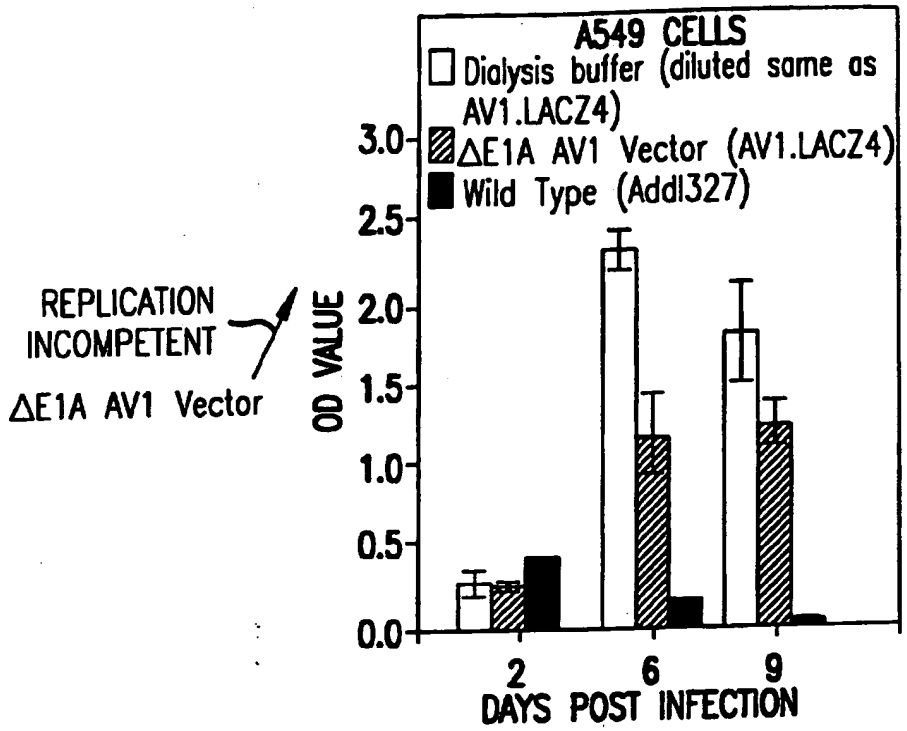

FIG.9C

CARCINOMA CELL LINE SPECIFIC KILLING (MOI=10)

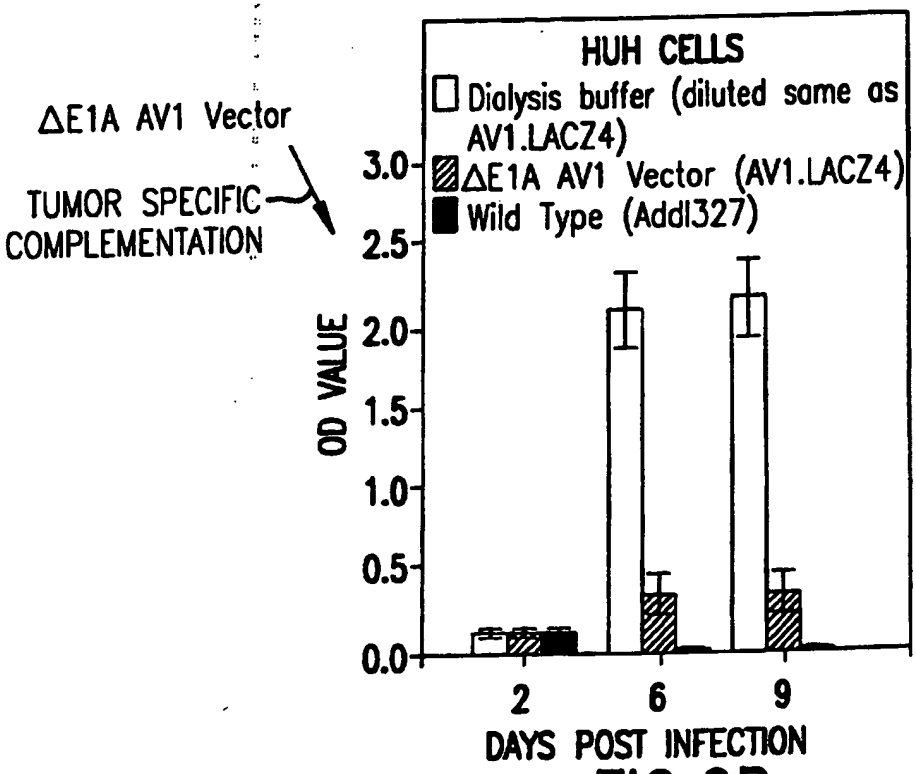

FIG.9D

TREATMENT GROUP	% ANIMALS FORMING TUMORS	% ANIMALS TUMOR FREE AT DAY 7	% ANIMALS TUMOR FREE AT DAY 20	% ANIMALS TUMOR FREE AT DAY 30
DILUENT (N=2)	100	0	0	0
DILUENT (N=2) PLUS GCV (300mg/kg/DAY)	100	0	0	0
AV1.NULL (N=2)	100	0	0	0
AV1.NULL (N=2) PLUS GCV (300mg/kg/DAY)	100	0	0	0
AV1.TK1 (N=6)	100	0	0	0
AV1.TK1 (N=6) PLUS GCV (300mg/kg/DAY)	100	100	50	50

FIG.10

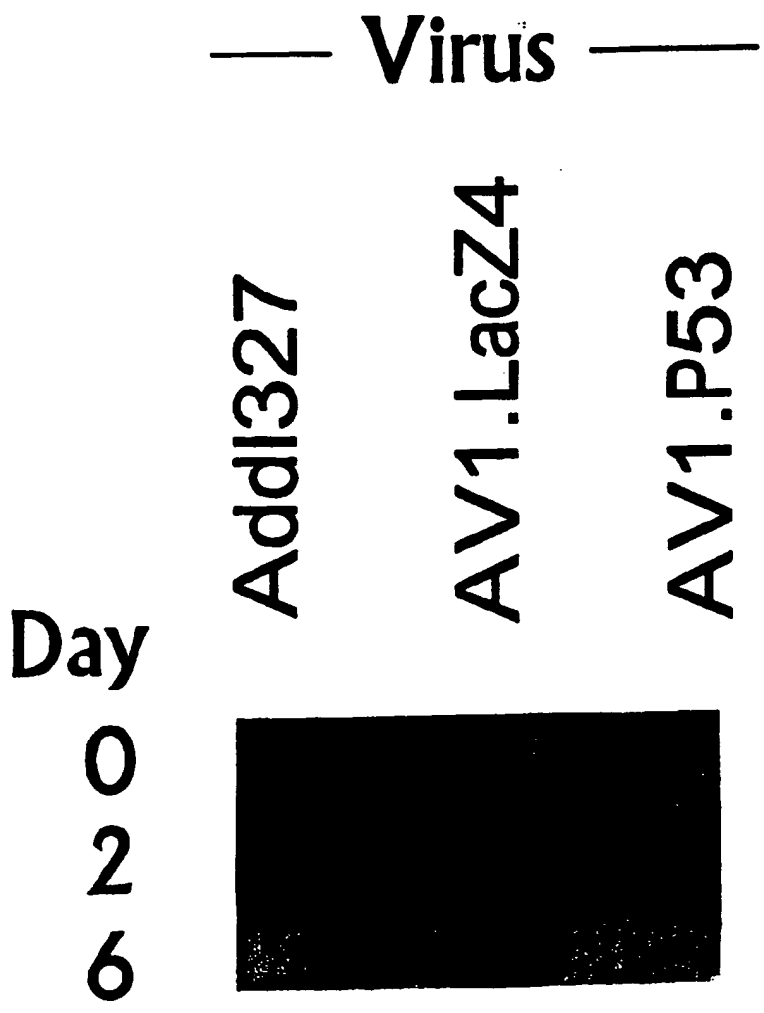

FIG. 11

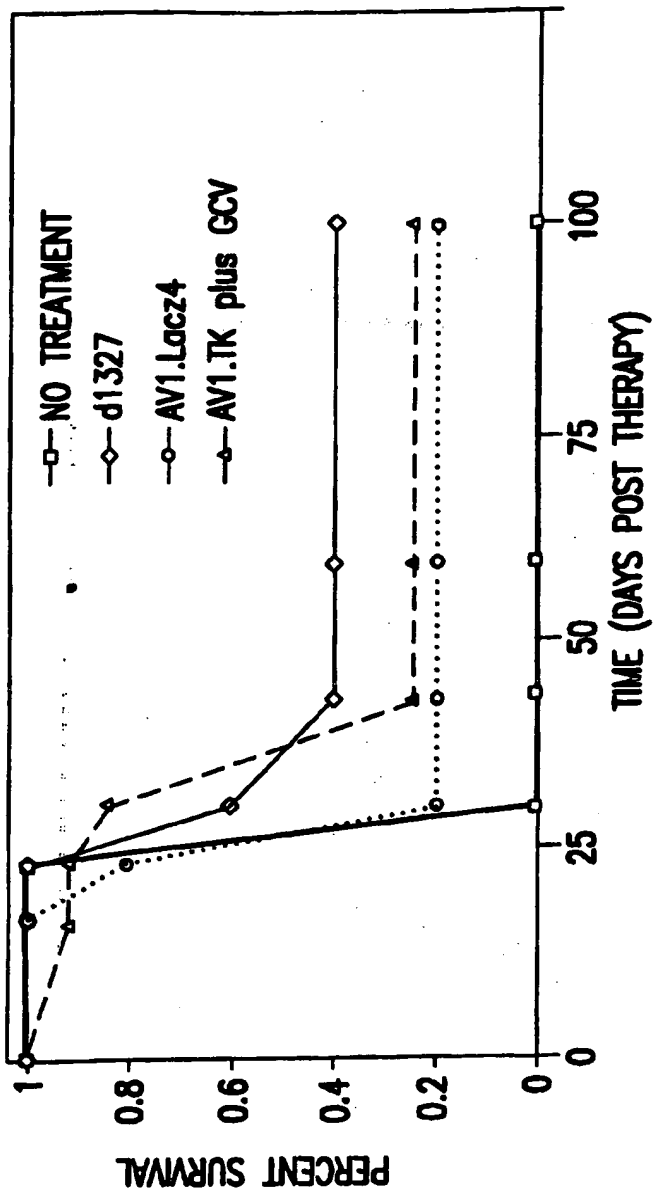
NO TREATMENT
d1327
AV1.Lacz4
AV1.TK plus GCV
PERCENT SURVIVAL
1
0.8
0.6
0.4
0.2
0
0
25
50
75
100
TIME (DAYS POST THERAPY)

FIG.12

INTERNATIONAL SEARCH REPORT

International application N .
PCT/US95/15431

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; C12N 15/00, 5/00
US CL : 514/44;435/320.1, 172.3, 240.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44;435/320.1, 172.3, 240.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ----- Y,P	WO, A, 95/12660 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 11 MAY 1995 (11.05.95), WHOLE ARTICLE, SPECIFICALLY PAGES 18-50.	1-13,15,18-23,25-27,29,32,33,35,38 ---------- 14,16,17, 24,28,34, 36, 37
Y	Oncogene, Vol. 9, issued September 1994, Shimizu et al. "RB Protein Status and Clinical Correlation from 171 Cell Lines Representing Lung Cancer, Extrapulmonary Small Cell Carcinoma, and Mesothelioma", pages 2441-2448, specifically page 2446.	14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
19 MARCH 1996

Date of mailing of the international search report
02 APR 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/15431

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Oncogene, Vol. 8, issued February 1993, Pulsieux et al. "p53 as a Growth Suppressor Gene in HBV-related Hepatocellular Carcinoma Cells", pages 487-490, specifically page 487.	16, 17, 24
X,P --- Y,P	WO, A, 95/11984 (CANJI, INC.) 04 May 1995 (04.05.95), Whole Article, specifically pages 33, 53-54.	1-13,15-17, 19-27, 32-38 --- 14,18
X	WO, A, 90/05180 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 17 May 1990 (17.05.90), pages 15,16,76-79.	30, 31
X,P	Cancer Research, Vol. 55, issued 15 November 1995, Kaneko et al. "Adenovirus-mediated Gene Therapy of Hepatocellular Carcinoma Using Cancer specific Gene Expression", pages 5283-5287, specifically page 5283.	13,16,17, 19-22, 24-28
Y	Cancer Research, Vol. 53, issued 15 September 1993, Fujiwara et al. "A Retroviral Wild-type p53 Expression Vector Penetrates Human Lung Cancer Spheroids and Inhibits Growth by Inducing Apoptosis", pages 4129-4133, specifically abstract and page 4129.	28

International Application No.
PCT/US95/15431

B. FIELDS SEARCHED
Electronic data basea consulted (Name of data basc and where practicable terms used):

APS
STN: MEDLINE, CAPLUS, CANCERLIT, BIOSIS, EMBASE
search terms: vector, transduction, transfection, virus, gene therapy, carcinoma, p53, rb.